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Late progression of renal pathology and cyst enlargement is reduced by rapamycin in a mouse model of nephronophthisis

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Because the size of renal cysts in the native kidneys of patients with ADPKD who have been transplanted was found to be reduced when rapamycin was the immunosuppressant, we tested the involvement of the mTOR pathway in cyst enlargement. Here, male *pcy* mice, with mutation in one of the nephronophthisis genes, were treated with rapamycin at an early (6 to12 weeks of age) or a later (20 to 30 weeks of age) disease stage by means of slow-release pellets containing placebo or rapamycin. Effectiveness of the rapamycin dose and delivery was shown by the inhibition of insulin-stimulated phosphorylation of p70S6K, a marker of mTOR activity, in skeletal muscle. Early treatment did not affect initial cyst development but when started late, there was a significant reduction in the rate of cyst enlargement, kidney fibrosis, and the progressive loss of renal function as measured by blood urea nitrogen. Kidneys of the mice treated through 30 weeks of age tended to be smaller and have less fibrosis compared with those of untreated or placebo-treated *pcy/pcy* mice at 20 weeks when treatment was initiated. Our study shows that rapamycin can prevent the late- but not the early-stage progression of renal pathology and deterioration of renal functional in this model of nephronophthisis, presumably by inhibiting mTOR activity.

Kidney International (2009) **76,** 178–182; doi:10.1038/ki.2009.147; published online 6 May 2009

KEYWORDS: mTOR; nephronophthisis; rapamycin; renal cysts

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Received 20 April 2008; revised 14 March 2009; accepted 24 March 2009; published online 6 May 2009

Nephronophthisis (NPHP) is part of a group of autosomal recessive conditions caused by mutations in one of six (or more, NPHP1-NPHP6) genes, similarly characterized by the presence of renal cysts with tubulointerstitial fibrosis.[1] NPHP is one of the hepatorenal fibrocystic conditions that also include; polycystic kidney diseases, Bardet-Beidl syndrome, Meckel-Gruber Syndrome, and Oral-Facial Digital Syndrome. NPHP is a leading cause of renal demise in the first three decades of life. Although the kidney is the major organ of interest in this condition, numerous other organs can be involved including: the liver (which can become fibrotic), eye (in Senior-Loken Syndrome), and brain (in Joubert Syndrome). The protein products of all of the NPHPs seem to localize to cilia;[1] however, the exact mechanism involved in the pathogenesis of these renal conditions are not well understood, but some data suggest that the cells are not correctly perceiving or responding to extracellular or environmental cues. The cellular pathways that have been implicated in the pathogenesis of these renal disorders include: G-protein-linked receptor stimulated cyclic AMP, altered cellular calcium and the mammalian target of rapamycin (mTOR) pathway.[2]

This study evaluated the possible involvement of the mTOR pathway in a murine model of NPHP using the mTOR inhibitor rapamycin (sirolimus). The murine *pcy* gene is orthologous to the human NPHP3 gene that causes adolescent NPHP.[3,4] The *pcy* mouse develops renal pathology[5] that is similar to that seen in NPHP. This model has been useful in evaluating other pathways that contribute to renal cystic conditions.[6] There is no treatment, at present, for NPHP, and as rapamycin is already available, we hoped to determine if mTOR inhibition is efficacious at inhibiting the initial development of renal cystic pathology and/or inhibiting the progression of the renal pathology and/or renal functional decline associated with this condition and other renal cystic diseases.

Rapamycin (or other mTOR inhibitors) has been shown to ameliorate the initial progression of renal cystic change in three non-orthologous rodent models of hepatorenal fibrocystic disease (the *Cy/+* rat,[7–9] the *bpk*, and *orpk* mice[10]). In addition, renal transplant patients with autosomal dominant

polycystic kidney disease (ADPKD) had less renal enlargement if rapamycin was part of their anti-rejection drug regimen[10] as well as a reduced liver volume.[11] These data clearly suggest that the mTOR pathway is involved in the progression of the renal cystic pathology. The mTOR pathways' activity potentially leads to the activation of the MEK–ERK pathway causing proliferation. Data from Omori *et al.*[12] found that the MEK–ERK pathway was increased in the *pcy* mouse. This study evaluated developmental aspects of mTOR activity and the efficacy of mTOR pathway inhibition in an orthologous model of human adolescent NPHP.

RESULTS

The cystic kidney of CD1-*pcy* mice with adolescent NPHP have elevated total Akt at 30 weeks and prominently elevated phosphorylated Akt at 12 and 30 weeks of age (Figure 1a and b). Akt is part of the pathways associated with activating mTOR. Thus, conditions are primed for mTOR activation. The best assessment of mTOR activity is the phosphorylation of p70S6K.[13] Total p70S6K is elevated in the cystic kidney at 12 and 30 weeks of age, whereas the proportion of phosphorylated p70S6K is slightly elevated at 4 weeks (over that seen in wild-type CD1 kidneys) with an age related increase at 12 and 30 weeks of age (Figure 1c and d). The very elevated (eightfold) P-p70S6K at 30 weeks is indicative of a strongly activated mTOR pathways. As with all form of PKD, phosphorylated ERK is elevated but rather uniformly across all ages as compared with wild-type CD1 mice (Figure 1e), suggesting that factors other than mTOR may be playing a stronger role early in the disease process.

Male CD1-*pcy* mice were treated with slow-release pellets containing rapamycin, which was released at an approximate rate of 3 mg/kg-bw/day. Treatment from 6 weeks of age to 12 weeks of age had no discernable effect on progression of the renal cystic disease during this early phase of the disease. The degree of renal enlargement between 6 weeks and 12 weeks was equivalent in both rapamycin and control groups (Table 1), suggesting that mTOR is not significantly involved in the initial cystic renal enlargement in murine adolescent NPHP. However, treatment from 20 to 30 weeks of age was efficacious in inhibiting the further renal cystic enlargement

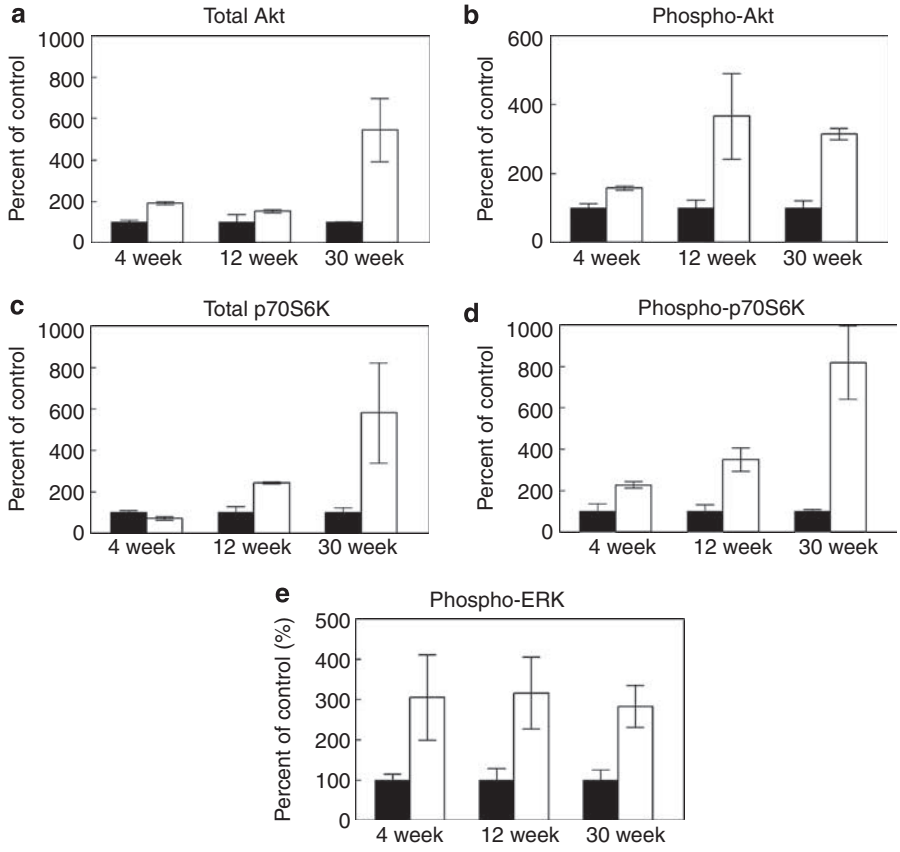

Figure 1 | Graphic representation of expression by western blot of Akt, p70S6K, and ERK in the *pcy* mouse kidney. (**a** and **b**) Total and phosphorylated Akt, which is upstream of mTOR activation. Total Akt protein in cystic kidney (open bars) is compared with comparably aged wild-type CD1 mice (black bars set at 100%) showing a dramatic increase in the cystic kidney at 30 weeks of age and the proportion of phosphorylated Akt is increased at both 12 and 30 weeks of age. (**c** and **d**) Total and phosphorylated p70S6K, which is downstream of mTOR and generally accepted as reflecting the activity of mTOR. Total p70S6K protein is increased in the cystic kidney (open bars) at both 12 and 30 weeks (normalized to age-matched wild-type controls, black bars). And phosphorylated p70S6K is increased in the cystic kidney at all ages, but is especially prominent late in the disease. (**e**) Phosphorylated ERK is elevated at all ages in the cystic kidney as has generally been found in all of the murine models of renal cystic disease. Bars (left to right) represent $n = 2, 3, 2, 2, 2, 3$.

Table 1 | Efficacy of rapamycin treatment

Group	6-Week control (n=6)	12-Week control (n=14)	6–12-Week rapamycin (n=7)	20-Week control (n=5)	30-Week control (n=10)	20–30-Week rapamycin (n=5)
BW (g)	18.2 ± 0.57	21.0 ± 0.20	22.9 ± 0.76*	19.9 ± 0.58	22.5 ± 0.57*	20.8 ± 1.55
KW (g)	0.46 ± 0.03	0.60 ± 0.02*	0.66 ± 0.07	0.79 ± 0.10	1.18 ± 0.06*	0.58 ± 0.10**
KW % BW	2.54 ± 0.13	2.85 ± 0.07	2.85 ± 0.25	3.95 ± 0.40	5.23 ± 0.27*	2.70 ± 0.28**, *
Cyst Vv (%)	ND	ND	ND	20.3 ± 2.8	33.4 ± 2.1*	17.4 ± 5.05**
Total cyst volume (cc)	ND	ND	ND	0.16 ± 0.03	0.40 ± 0.035*	0.10 ± 0.04**
Cyst vol % BW ($\times 10^{-3}$)	ND	ND	ND	8.1 ± 1.4	17.6 ± 1.5*	5.0 ± 1.9**
Fibrosis score	ND	ND	ND	2.1 ± 0.2	2.7 ± 0.2	1.6 ± 0.2**
BUN (mg/100ml)	17.2 ± 1.3	17.3 ± 0.6	17.4 ± 1.0	24.8 ± 1.9	32.2 ± 2.2*	22.9 ± 1.4**

BUN, blood urea nitrgen; BW, body weight; Cyst Vv, cyst volume density; ND, not done; KW, kidney weight.
Mean ± s.e.m.
*$P \leqslant 0.05$ for difference from treatment initiation age (6- or 20-week values).
**$P \leqslant 0.05$ for difference from age-matched control.

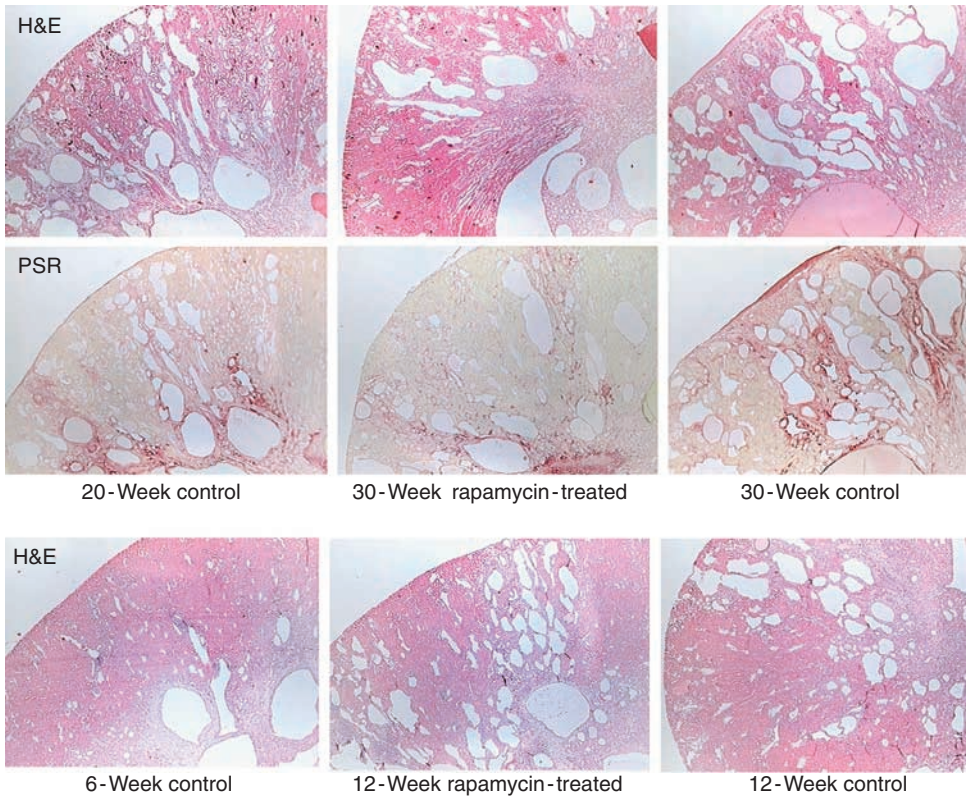

Figure 2 | The kidneys from 30-week rapamycin-treated *pcy* mice exhibited less histopathological cystic change and fibrosis (Picrosirius red (PSR) sections) than did 30-week control cystic kidney. There was generally less prominent renal cystic change and fibrosis in the 30-week-treated kidney than in 20-week kidneys; however, generally these did not meet statistical significance. There was little, if any, difference seen between the 12-week rapamycin-treated and 12-week control kidneys. H&E, hematoxylin and eosin stain.

and deterioration of renal function (Table 1). The renal pathology in the 30-week control group was generally increased compared with the 20-week control, whereas the 30-week rapamycin-treated group was less severely affected compared with the 30-week control group (Figure 2). The 30-week histological specimens were evaluated for mitosis and apoptosis in the cystic epithelial cells. Whereas there was a slight tendency for decreased cystic epithelial cell proliferation in the rapamycin-treated mice, there were no significant differences between rapamycin and control kidney (mitotic index (%), rapamycin vs control, 0.15% ± 0.15 vs 0.50% ± 0.15 and apoptosis (%) 3.10% ± 0.67 vs 2.45% ± 0.63). The use of rapamycin was effective in halting the progression of the histopathology and renal functional deterioration in this later stage of the disease. The kidneys in the rapamycin-treated group were actually smaller (when expressed as a percentage of total body weight) than those of the 20-week control group, whereas total body weight was not different,

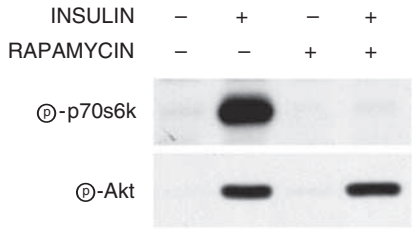

Figure 3 | Immunoblots for phosphorylated PKB (Akt) and p70S6K were carried out in insulin-stimulated skeletal muscle to evaluate the effect of the rapamycin slow-release pellets and their dosage of rapamycin (approximately 3 mg/kg/day) on mTOR activation. As expected, insulin induced activation of PKB, regardless of the presence or absence of rapamycin, as it is upstream from mTOR. Insulin-activated mTOR activity as evinced by phosphorylated p70S6K, but only in the absence of rapamycin, indicating that the rapamycin pellets successfully blocked activation of mTOR and its downstream targets (for example, p70S6K). Using an insulin-stimulated skeletal muscle system, the rapamycin slow-release pellets were shown to be effective at inhibiting a stimulated mTOR pathway.

suggesting that treatment was able to cause some limited degree of disease regression.

The use of slow release pellets containing the rapamycin were very effective at inhibiting specifically stimulated mTOR system. Rapamycin inhibited insulin stimulated phosphorylation of p70S6K in muscle without affecting the amount of upstream phosphorylated AKT in wild-type mice (Figure 3). The muscle system was used to evaluate the effectiveness of the rapamycin pellets to avoid potential complications associated with variability seen in this disease.

DISCUSSION

Nephronophthisis, although not as prevalent as ADPKD, is an important cause of renal failure in the pediatric population. Adolescent NPHP causes renal demise in adolescence and to date there is no intervention for this condition. This study found that rapamycin intervention in the early stage of the disease did not inhibit the initial development of cystic pathology; however, treatment with rapamycin was efficacious in the later stage. At 20 weeks of age, renal cystic pathology and fibrosis were already significantly developed, yet rapamycin was able to inhibit further pathology and functional deterioration. There was even evidence that late-stage treatment caused some regression of the existing pathology present at 20 weeks of age. By 30 weeks, the cystic pathology and function deterioration had progressed to a point where the efficacy of this treatment could be clearly identified.

Rapamycin and/or other inhibitors of the mTOR pathway have been shown to be efficacious in three different rodent models, the *Cy/+* rat,[7–9] the *bpk*, and *orpk* mice.[10] These interventions were generally targeting the initial stages of the cystic disease. The retrospective evaluation of renal transplant patients with ADPKD who received rapamycin/sirolimus as part of their immunosuppressive treatment also showed inhibition of both renal and liver pathology.[10,11] These retrospective evaluations were clearly at late stages of the ADPKD. Currently, there are prospective clinical trials using mTOR inhibitors for the treatment of ADPKD (see Polycystic Kidney Disease Foundation website on clinical trials, http://www.pkdcure.org/). The determination of the efficacy of rapamycin (or any treatment that will be proposed for clinical trials) in orthologous rodent models of renal cystic disease is important for determining at what stage(s) of the disease the treatment would be most effective. Data currently indicate that Octreotide, a somatostatin agonist, which is also in clinical trials for PKD, may act through the same pathway as mTOR inhibitors,[13] suggesting that the mTOR pathway may be part of the final common pathway involved in PKD progression. To that end, this study has provided evidence that the mTOR inhibitor rapamycin is efficacious in already established renal cystic pathology of the *pcy* mouse model of NPHP, but not effective in the early stage of this rodent form of renal cystic disease.

Most interventions that have been evaluated in rodent models are aimed at the initial stages of the cystic disease; however, many of the affected patients may not even be diagnosed until they already have significant pathology, being more analogous to our 20-week *pcy* mouse. The identification of interventions that are efficacious at more advanced stages of the renal cystic disease could be very valuable. Although it is evident that some aspects of the renal pathology were already well established when treatment was initiated and may be irreversible, it also seems that some aspects of the pathology are not only stopped, but may even be reversed with treatment. This seems evident from our pcy mouse study and is also suggested by retrospective human ADPKD studies.[10,11] A similar reversal of renal cystic pathology in rodents was observed with one other treatment, vasopressin V2 receptor antagonism, in the *pcy* mouse.[6] However, the prospect of reversing later stage renal and liver cystic disease may ultimately be the goal of any treatment for these conditions.

MATERIALS AND METHODS

Studies were conducted at the Indiana University School of Medicine with a colony of CD1-*pcy*/*pcy* mice and all the procedures were approved by the IACUC. Male *pcy*/*pcy* and age-matched normal CD1 mice were used in this study. Western blot was used to evaluate activity of the mTOR pathway in pcy mice, as compared with the age-matched wild-type CD1 mice. Total and phosphorylated Akt and p70S6K, as well as phosphorylated ERK (known to be elevated in the cystic kidney, especially in the cysts[12]) were evaluated. The mice were anesthetized (sodium pentobarbital 100 mg/kg), kidneys were removed, snap frozen in liquid nitrogen, homogenized, measured for total protein, and equal amounts of protein were electrophoresed in acrylamide gels. Immunoblots for total and phosphorylated PKB (Akt), and p70S6K as well as phosphorylated ERK were carried out to evaluate activation of the mTOR pathway. The primary antibodies used for western blots were from Upstate Biotechnology (LakePlacid, NY, USA) and the peroxidase secondary antibody was from Vector Laboratories (Burlingame, CA, USA).

As it was apparent that the mTOR pathway was activated in the *pcy* mouse, the efficacy of rapamycin to inhibit progression of the renal cystic disease was assessed. Mice were either started on treatment at 6 weeks of age (and terminated at 12 weeks to assess the ability to slow the initial development of cystic renal enlargement) or 20 weeks of age (and terminated at 30 weeks to assess the ability of the treatment to slow, or stop cystic pathology progression after it was already well established). Treatment was accomplished using slow-release pellets implanted subcutaneously (approximately 3 mg/kg/day in 45 day slow-release pellets, Innovative Research of America, FL, USA). Control groups included: (a) 6-week controls, (b) 12-week controls, (c) 12-week rapamycin-treated, (d) 20-week controls, (e) 30-week controls, (f) 30-week rapamycin-treated. As data from the placebo-implanted and sham-implanted values were not different, those groups were combined for 12- and 30-week control groups. Mice were provided food (Teklad 7002, Harlan Lab, Indianapolis, IN, USA) and water *ad libitum* and maintained on a 12 h:12 h, dark:light cycle. At the time of killing killing, the mice were weighed, anesthetized (sodium pentobarbital 100 mg/kg given i.p.), and the blood was collected. A laparotomy was carried out and kidneys were flushed with saline. The left kidney was weighed and then frozen in liquid nitrogen. The right kidney was perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, weighed, and transverse sections were further immersion-fixed before processing for paraffin embedment. Sera was assayed for urea nitrogen (Sigma Urea Assay kit #640, Sigma Aldrich, St Louis MO, USA). Paraffin sections of the kidney were stained (hemotoxylin/eosin or Picrosirius red), and random regions of the cortex/outer medulla were photographed. The amount of cystic change (cyst volume density (Vv)) was determined from these random fields using point count stereology methods. The total cyst volume was determined by multiplying cyst Vv (cc/cc) times the kidney weight. Cyst volume as a percentage of total body weight was determined by dividing the total cyst volume by total body weight. Fibrosis was determined from 10–15 random fields on Picrosirius red-stained slides using a 1+ to 4+, minimal-to-severe, qualitative score. The cystic epithelia were evaluated for proliferation (mitotic cell index using morphological criteria for metaphase through telophase) and apoptosis (apoptotic bodies) and expressed as a percentage. Data were analyzed using analysis of variance for the group and, if significant, individual comparison was made using analysis of variance. Graphed and table data are expressed as mean ± standard error of the mean (s.e.m.).

To confirm the efficacy of the dose of rapamycin in the slow-release pellets, the mTOR pathway was stimulated with insulin, and the phosphorylation/activation of upstream and downstream components were assessed using western blots in the skeletal muscle. Briefly, wild-type mice with the slow-release rapamycin pellets (implanted 2 weeks earlier) were injected i.p. with either insulin or saline, 15 min before killing, and then anesthetized and tibialis anterior muscle was removed, snap frozen in liquid nitrogen, homogenized, measured for total protein, and electrophoresed in acrylamide gels. Immunoblots for phosphorylated Akt, and p70S6K were performed to evaluate activation of the mTOR pathway. As expected, insulin induced activation of Akt, regardless of the presence or absence of rapamycin. Insulin also activated the downstream component of mTOR, phosphorylating p70S6K, but only in the absence of rapamycin. The slow-release pellets of rapamycin successfully blocked the activation of mTOR, evidenced by the lack of phosphorylation of the downstream target (for example, p70S6K). The skeletal muscle system was used, as we could activate the mTOR pathway with insulin and show that rapamycin could not inhibit this activation, independent of any changes associated with the disease condition.

Note in proof

An MRI study on the efficacy of rapamycin in *pcy* mice, treated from 18 weeks to 28 weeks, is currently 'in press'[14] and confirms rapamycin inhibition of renal cystic disease, even at this later stage of disease in this model of NPHP.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

An abstract, which presented some of these data was presented during the Renal Week 2007 meeting of the American Society of Nephrology.

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