

Original Article

Inhibition of mTOR with sirolimus slows disease progression in Han:SPRD rats with autosomal dominant polycystic kidney disease (ADPKD)

Patricia R. Wahl¹, Andreas L. Serra^{1,2}, Michel Le Hir³, Klaus D. Molle⁴, Michael N. Hall⁴ and Rudolf P. Wüthrich^{1,2}

¹Physiological Institute, ³Anatomical Institute, University Zürich-Irchel, ²Division of Nephrology, University Hospital, Zürich and ⁴Division of Biochemistry, Biozentrum, University of Basel, Switzerland

Abstract

Background. Autosomal dominant polycystic kidney disease (ADPKD) is characterized by dysregulated tubular epithelial cell growth, resulting in the formation of multiple renal cysts and progressive renal failure. To date, there is no effective treatment for ADPKD. The mammalian target of rapamycin (mTOR) is an atypical protein kinase and a central controller of cell growth and proliferation. We examined the effect of the mTOR inhibitor sirolimus (rapamycin) on renal functional loss and cyst progression in the Han:SPRD rat model of ADPKD.

Methods. Five-week-old male heterozygous cystic (Cy/+) and wild-type normal (+/+) rats were administered sirolimus (2 mg/kg/day) orally through the drinking water for 3 months. The renal function was monitored throughout the treatment phase, and rats were sacrificed thereafter. Kidneys were analysed histomorphometrically, and for the expression and phosphorylation of S6K, a well-characterized target of mTOR in the regulation of cell growth.

Results. The steady increase in BUN and creatinine in Cy/+ rats was reduced by 39 and 34%, respectively with sirolimus after 3 months treatment. Kidney weight and 2-kidney/total body weight (2K/TBW) ratios were reduced by 34 and 26% in sirolimus-treated Cy/+ rats. Cyst volume density was also reduced by 18%. Of importance, Cy/+ rats displayed enhanced levels of total and phosphorylated S6K. Sirolimus effectively reduced total and phosphorylated levels of S6K.

Conclusion. We conclude that oral sirolimus markedly delays the loss of renal function and retards cyst development in Han:SPRD rats with ADPKD.

Our data also suggest that activation of the S6K signalling pathway plays an important role in the pathogenesis of PKD. Sirolimus could be a useful drug to retard progressive renal failure in patients with ADPKD.

Keywords: ADPKD; Han:SPRD rats; mTOR; rapamycin; sirolimus; S6K

Introduction

Autosomal-dominant polycystic kidney disease (ADPKD) is the most common form of renal cystic disease, affecting all ethnic groups worldwide, with an incidence of 1 in 500 to 1 in 1000. The disease accounts for up to 10% of all patients requiring renal replacement therapy. Affected individuals usually present in the 3rd and 4th decade of life, progressing to end-stage renal disease (ESRD) within 5–10 years after the development of renal insufficiency [1].

The disease is characterized by the progressive development of fluid-filled cysts in the kidney. The renal cysts originate from the epithelia of the nephrons and are lined by a single layer of cells that have a higher rate of cellular growth and proliferation. Various abnormalities in gene expression, fluid secretion, apoptosis and extracellular matrix production have been described in ADPKD [2]. PKD1 and PKD2 encode for the proteins polycystin-1 and polycystin-2, and various mutations in these genes are linked to the occurrence of ADPKD [3–6]. How defects in these genes lead to the massive cyst growth in patients with ADPKD is being investigated [7].

Currently, there is no specific treatment for PKD other than supportive care and blood pressure control. Usually dialytic treatment or renal transplantation

Correspondence and offprint requests to: Prof. Dr Rudolf P. Wüthrich, Renal Division, University Hospital, Rämistrasse 100, 8091 Zürich, Switzerland. Email: rudolf.wuethrich@usz.ch

becomes necessary when the disease has progressed to end-stage renal failure. There is significant interest in searching for specific drugs which decrease or halt the progression of cyst development, thus slowing renal functional deterioration and preventing the development of ESRD [8–10].

The Han:SPRD rat is a well-known model of ADPKD, although the gene defect has not been identified yet [11]. Recently, the drug rapamycin (sirolimus) – an inhibitor of the mammalian target of rapamycin (mTOR) – was found to significantly inhibit cyst growth in the Han:SPRD rat [12]. The protein kinase mTOR has emerged as a major effector of cell growth and proliferation via the regulation of protein synthesis. mTOR controls protein synthesis through a number of downstream targets, some of which are phosphorylated directly by mTOR, while others are phosphorylated indirectly, and could be dysregulated in ADPKD.

The mTOR inhibitor sirolimus is an immunosuppressant drug with antiproliferative and growth inhibiting effects [13,14]. It is used clinically in recipients of renal allografts to prevent transplant rejection. It is also used in coated stents to prevent coronary artery re-stenosis after angioplasty [15]. Very recently, sirolimus has shown clinical effectiveness in kidney transplant recipients with Kaposi's sarcoma [16]. We hypothesized that mTOR and its downstream cell growth regulatory proteins are dysregulated in PKD, and that sirolimus could be an effective oral drug to inhibit cyst progression. We therefore used the Han:SPRD rat model of ADPKD [11] to test the effect of oral sirolimus (Rapamune®) on loss of renal function and cyst progression. We also examined the activity of the mTOR downstream target S6K (p70^{S6K}) [17] in these ADPKD rats and tested the effect of sirolimus on this effector protein.

Subjects and methods

Animals

The study was conducted in heterozygous (Cy/+) and normal littermate control (+/+) Han:SPRD rats. Only male rats were used since the disease progresses faster in male compared with female rats. A colony of Han:SPRD rats was established in our animal care facility from breeding pairs that were obtained from the Rat Resource & Research Center (Columbia, MO, USA). The study protocol was approved by the regulatory commission for animal studies, a local government agency. Rats had free access to tap water and standard rat diet.

Study drug

Rapamune oral solution (1 mg/ml) was kindly provided by Wyeth-AHP (Schweiz) AG, Switzerland.

Experimental protocol

Male Cy/+ and +/+ rats were weaned and were then treated at 5 weeks of age ($n=12$) with 2 mg/kg/day sirolimus.

Additional male Cy/+ and +/+ rats ($n=16$) were not treated, and they served as controls. The drug was administered in the form of Rapamune® oral solution (Wyeth) in the drinking water for 3 months. Drinking bottles were protected from light with aluminum foil and were changed every 2 days. The concentration of sirolimus (Rapamune® oral stock solution 1 mg/ml) was adjusted according to rat body weight. Blood was drawn from the tail veins every month. After 3 months treatment, rats were anaesthetized with isoflurane, and kidneys were removed, decapsulated and weighed. Slices of each kidney were preserved in 4% paraformaldehyde and embedded in paraffin for histological examinations. Additional slices were snap frozen in liquid nitrogen and used for protein extraction.

Blood chemistry

Blood was collected in lithium-heparin tubes and centrifuged at 4°C. Plasma was stored at –20°C, and was analysed later for blood urea nitrogen (BUN) and creatinine using kinetic color test and the modified Jaffé method, respectively. Blood for sirolimus levels was collected under the same conditions, but using EDTA tubes and analysed using HPLC-mass spectroscopy. All samples were analysed at the Institute for Clinical Chemistry, University Hospital (Zürich, Switzerland).

Histology

Mid-transversal slices of the kidney, about 2 mm-thick, were fixed by immersion in phosphate-buffered 4% paraformaldehyde, dehydrated in alcohol series and embedded in paraffin. Three micrometer thick sections were stained with the periodic acid-Schiff (PAS) technique. The cyst volume density (CVD) was evaluated in these sections by morphometry, using the technique of point counting [18].

To avoid subjective sampling biases, micrographs for morphometry were taken according to an invariable protocol. At the magnification used (Zeiss Plan-Neofluar 10×/0.30), the diameter of the field of view was close to the thickness of the renal cortex in healthy rats. The first micrograph was taken as the field was positioned on one extremity of the horseshoe-shaped cortex. For further sampling, the microscope stage was moved clockwise by steps of one field diameter, while the surface of the kidney remained at the limit of the field. A micrograph was made at every second step. A total of 4–6 micrographs was obtained for one section. One section was evaluated per rat. The pixel size of the micrographs was 1300 × 1030 (1.34 μm/pixel). The cyst volume density of cysts was evaluated by point counting. An orthogonal grid with a line spacing of 150 pixels was used. The person responsible for morphologic documentation and for morphometry was blinded to the experimental groups.

Analysis of S6K activity

The amount of total and phosphorylated S6K was assessed by immunoblotting, using specific antibodies. Samples of kidney tissue were snap-frozen in liquid nitrogen and stored at –80°C. On the day of immunoblotting, ice-cold lysis buffer was prepared. The lysis buffer consisted of 50 mM Tris base, pH 7.4; 150 mM NaCl; 1% deoxycholate; 1% SDS; and 1% Triton X-100. Immediately before use, the lysis buffer was

supplemented with 1 mM PMSE, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and protease and phosphatase inhibitor cocktail. Each sample was homogenized in this buffer with a Dounce homogenizer. The protein concentration of each sample was measured.

SDS-PAGE sample buffer was added to the extracts. Extracts were heated to 90°C for 5 min for denaturation. A total of 30 μ g protein was loaded per lane for standard SDS-PAGE (7.5%), and Western analysis. The following antibodies were used for detection: sc-230 (St Cruz Biotechnology, Inc., Santa Cruz, CA, USA; detects S6K), #9205 and #9204 (Cell Signaling Technology, Inc., Beverly, MA, USA; detect S6K phosphorylated on Thr389 and Thr 421/Ser424, respectively), and MAB 1501 (Chemicon, Temecula, CA, USA; detects actin). For signal detection, horseradish peroxidase-conjugated secondary antibodies and SuperSignal Femto/Pico ECL kits were used (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

Data are presented as means \pm SD and were analysed by one-way ANOVA with post-test according to Newman-Keuls. A *P*-value of <0.05 was considered significant.

Results

Judging by physical appearance, the treatment with sirolimus was well tolerated. None of the 12 sirolimus-treated rats died, and all 16 control rats survived. Sirolimus blood levels were obtained in all treated animals, and were between 0.5 and 1.9 μ g/l. Figure 1 shows that body weight increased steadily in untreated and sirolimus treated Cy/+ and +/+ rats. Treatment with sirolimus did not significantly change body weight after 3 months of treatment (−3.3% in +/+ and −11% in Cy/+ rats).

Renal functional loss is inhibited with sirolimus

Figure 2 demonstrates that the BUN and creatinine levels remained within normal limits in +/+ rats but increased progressively in untreated Cy/+ rats. BUN and creatinine levels also increased in sirolimus-treated Cy/+ rats, but to a lesser degree. After 3 months of treatment, the average BUN level was 39% less in sirolimus-treated Cy/+ rats (*n*=7) compared with untreated Cy/+ rats (*n*=9) (45.6 ± 4.2 vs 75.3 ± 16.9 mg/dl; *P*<0.001), and creatinine levels were 34% less (0.89 ± 0.13 vs 1.34 ± 0.24 mg/dl; *P*<0.001). This demonstrates that sirolimus effectively inhibited the loss of renal function in male Han:SPRD rats.

Renal weight and cyst volume density are reduced with sirolimus

Figure 3 shows that the increase in kidney weight was effectively reduced in sirolimus-treated Cy/+ compared with untreated Cy/+ rats. Treatment with sirolimus resulted in a 34% lower kidney weight in Cy/+ rats. Sirolimus treatment did not have a significant effect on kidney weight in wild-type +/+ rats (Figure 3A). We also determined the 2K/TBW ratios. Figure 3B demonstrates that sirolimus reduced the 2K/TBW ratio by 26% in Cy/+ rats.

Histological examination of the Han:SPRD kidneys revealed a significant reduction in cyst size in sirolimus-treated Cy/+ rats compared with untreated Cy/+ animals (Figure 4). A more detailed microscopical examination revealed the presence of tubular cysts in the cortex, and to a lesser extent in the outer medulla of untreated Cy/+ kidneys. The inner medulla was not affected. Cystic expansion of Bowman's capsule was observed in a few glomeruli. Mononuclear cell infiltrates were observed focally in the peritubular interstitium. This histopathological pattern was not

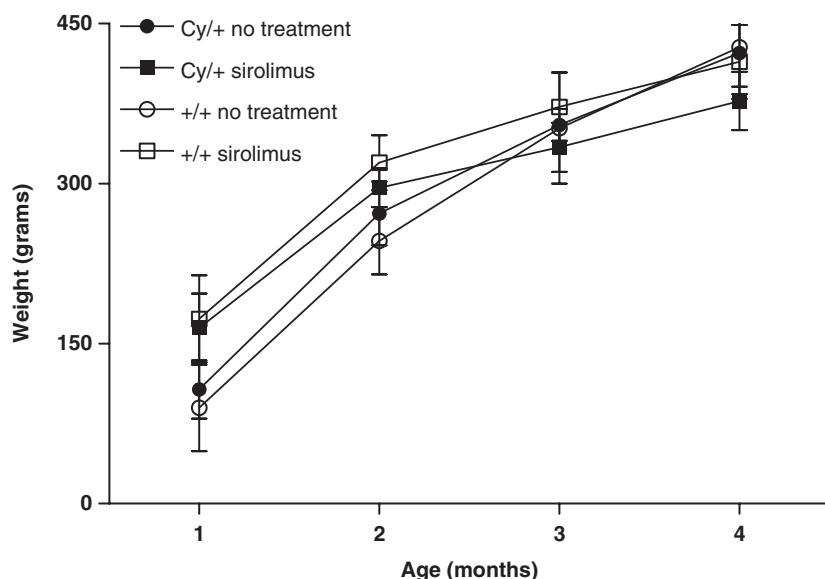


Fig. 1. Weight gain (g) in the four animal groups at 1, 2, 3 and 4 months of age. Treatment with sirolimus was started at 5 weeks and did not significantly influence weight gain (ANOVA).

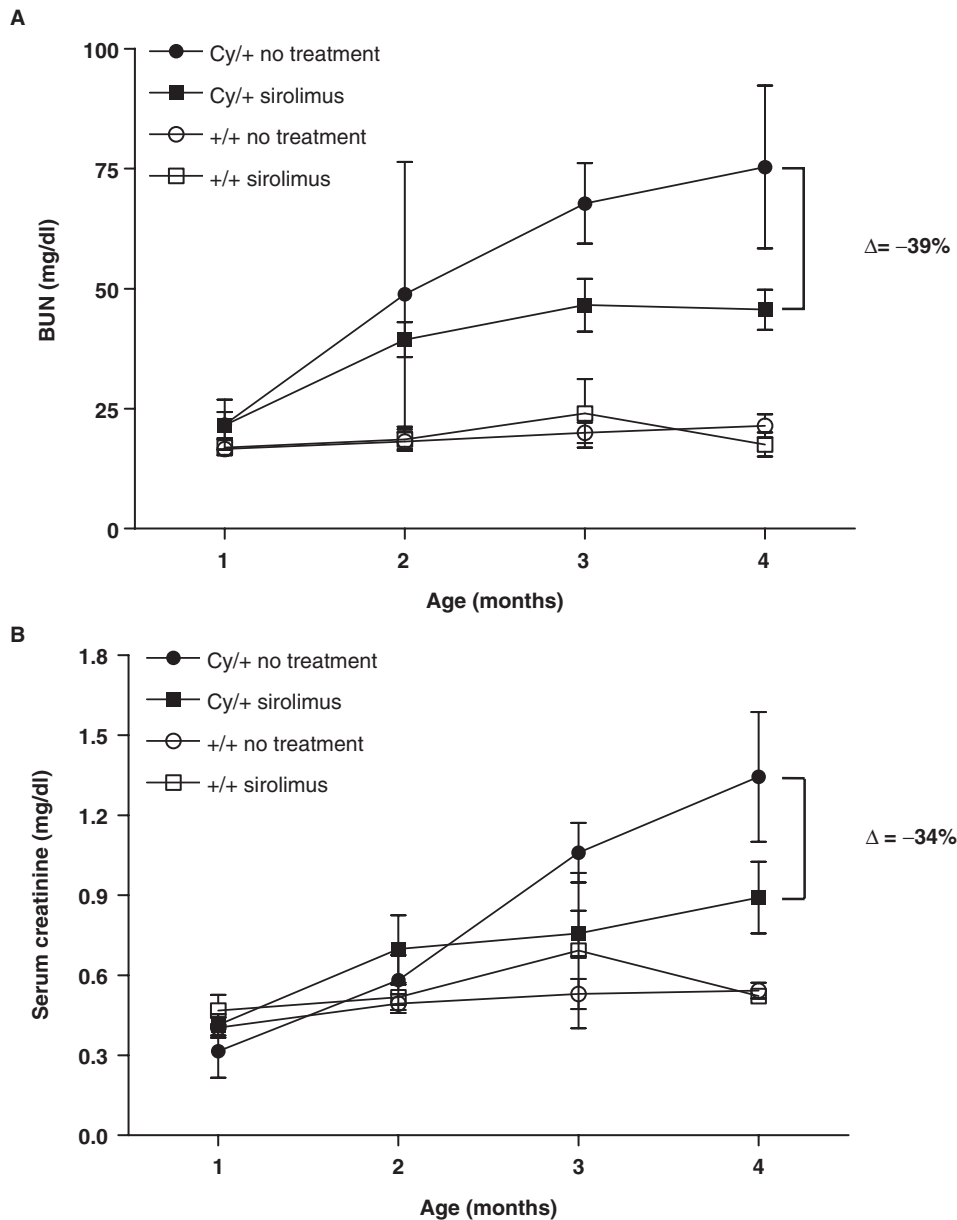


Fig. 2. Blood urea nitrogen (BUN) (A) and serum creatinine (B) levels were measured monthly in all rats. The rise in BUN and creatinine was significantly reduced by sirolimus in Cy/+ rats.

affected qualitatively by sirolimus treatment in Cy/+ kidneys. Untreated and sirolimus-treated wild-type +/+ kidneys showed no difference in histology (data not shown).

The most significant effect of sirolimus on kidney histology was a significant reduction of the cyst volume density in the cortex of Cy/+ rats. Figure 5 demonstrates that cyst volume density was 18% less in sirolimus-treated Cy/+ rats ($P < 0.001$). Since the reference volume for evaluation of volume density was the total cortical volume and since sirolimus treatment considerably reduced the increase of kidney weight in Cy/+ rats, the effect of sirolimus on the cyst volume density certainly underestimates its effect on the absolute volume of cysts.

Total and phosphorylated levels of S6K are enhanced in ADPKD rats

mTOR controls protein synthesis and cell growth by phosphorylating and thereby activating the 70 kDa ribosomal S6 kinase (S6K) [19]. Thus far it has not been tested whether mTOR-S6K signalling is altered in ADPKD, and whether the mTOR inhibitor sirolimus affects ADPKD. We therefore examined S6K expression and phosphorylation in untreated and sirolimus-treated Cy/+ rats. Figure 6 shows that total levels of S6K are markedly increased in the kidneys of 4-month-old Cy/+ Han:SPRD rats compared with wild-type +/+ rats. The level of expression of S6K was effectively reduced in sirolimus-treated Cy/+ rats.

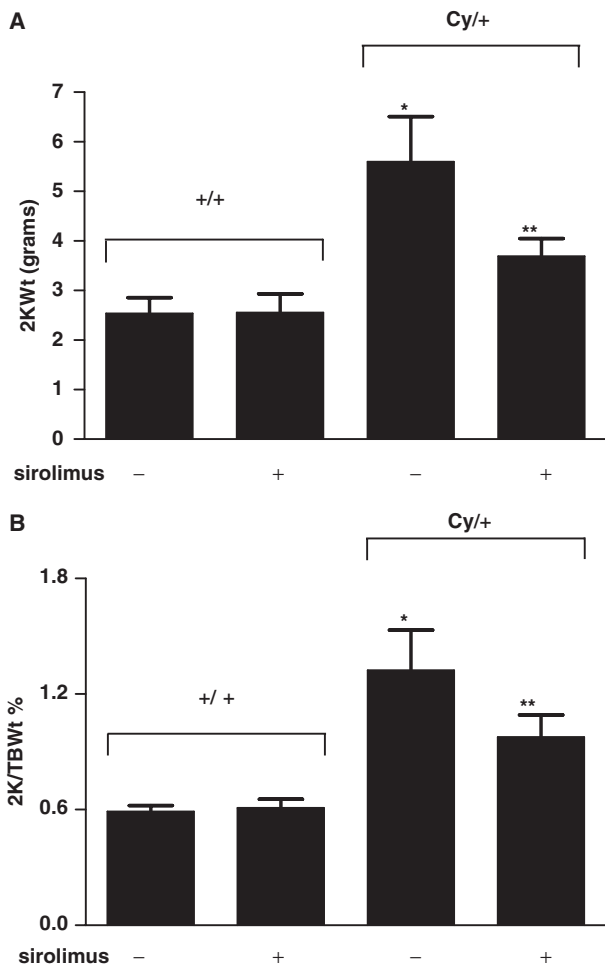


Fig. 3. The 2-kidney weights (2Kwt) (A) and 2-kidney/total body weight (2K/TBWt) ratios (B) were determined after 3 months of treatment with sirolimus. Both parameters were significantly reduced by sirolimus in Cy/+ rats. * $P < 0.001$ (Cy/+ untreated vs +/+ untreated); ** $P < 0.001$ (Cy/+ untreated vs Cy/+ sirolimus-treated).

We then measured the activity of S6K by analysing the mTOR-dependent phosphorylation of S6K at threonine 389 and threonine 421/serine 424 [20–23]. Figure 5 shows that the phosphorylated forms of S6K were undetectable in normal +/+ kidneys, but markedly enhanced in Cy/+ kidneys. Sirolimus effectively reduced phosphorylation at Thr389 and Thr421/Ser424 of S6K in Cy/+ kidneys. Overall, there was an excellent correlation between the histomorphometric findings and the degree of S6K phosphorylation.

Discussion

The massive and spontaneous development of cysts results in progressive deterioration of renal function in male Han:SPRD rats. This leads to symptomatic renal failure and premature death in these animals. Here, we demonstrate that sirolimus, when given orally for 3 months, significantly improves renal function in male Han:SPRD rats. Sirolimus treatment effectively

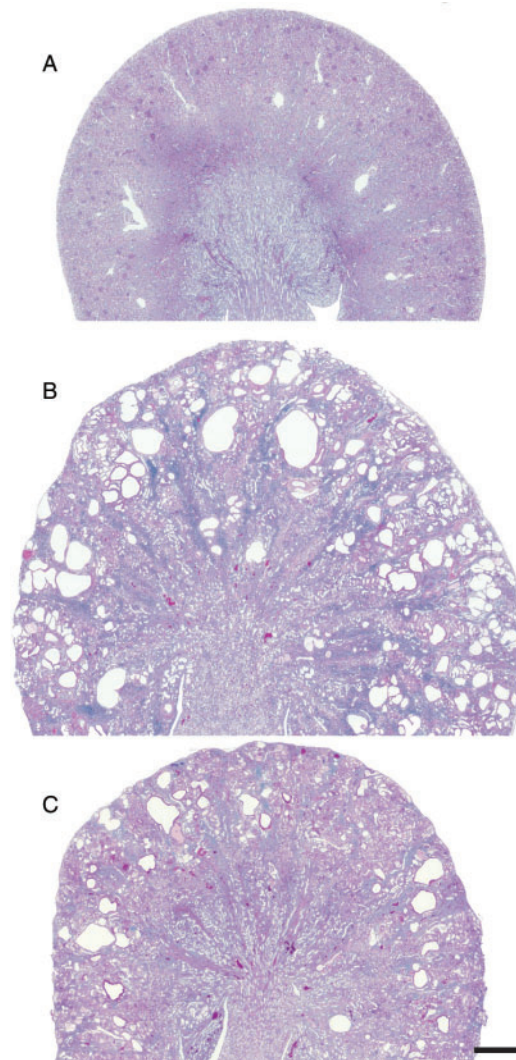


Fig. 4. Paraffin sections of kidneys of a wild-type, sirolimus-treated (A), of a Cy/+ untreated (B) and of a Cy/+ sirolimus-treated rat (C). Sirolimus reduced the kidney size, cyst volume and cyst density markedly in Cy/+ rats. PAS staining. Bar length: 1.5 mm.

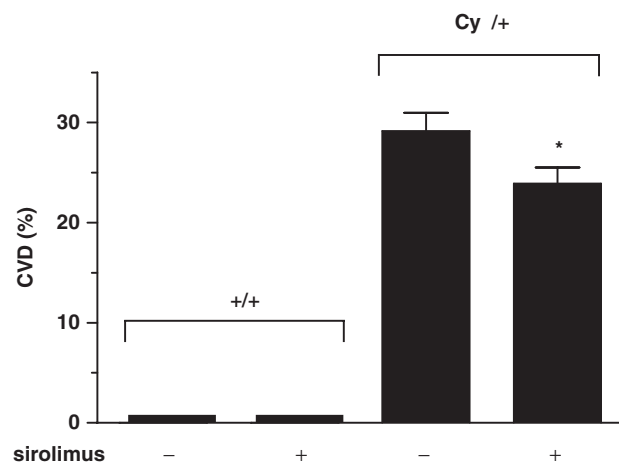


Fig. 5. Cyst volume density (CVD) was massive in Cy/+ rats and was significantly decreased (–18%) in sirolimus-treated Cy/+ rats. * $P < 0.001$ (Cy/+ untreated vs Cy/+ sirolimus-treated).

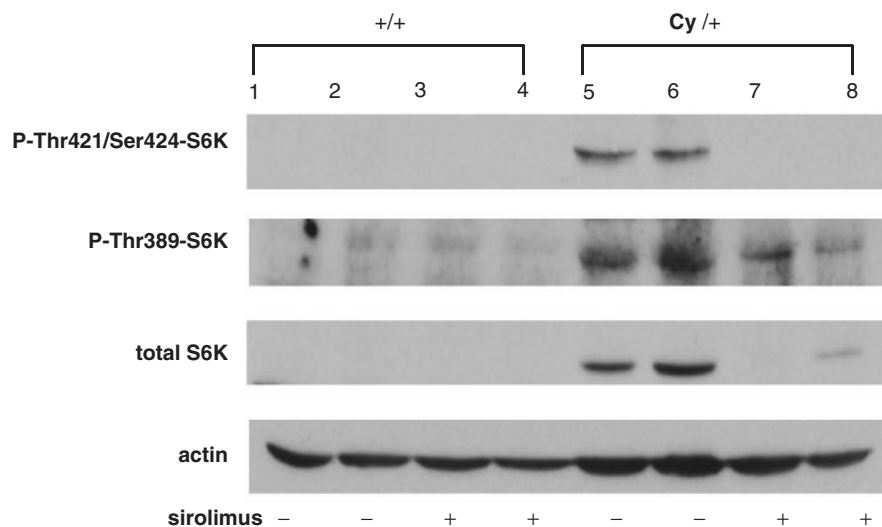


Fig. 6. SDS-PAGE and Western blot analysis from kidney protein extracts for total amounts of S6K, phosphorylated S6K (Thr389, and Thr421/Ser424) and actin. Two representative animals were analyzed from each group. The amount of total and phosphorylated S6K was markedly increased in *Cy/+* kidneys (lanes 5 and 6), and this was effectively suppressed by sirolimus (lanes 7 and 8).

controlled the massive increase in kidney weight in *Cy/+* rats and significantly reduced the cyst volume density. Our data confirm and extend a very recent report by Tao *et al.* [12]. They found that rapamycin, when injected intraperitoneally for 5 weeks, improved renal function and slowed cyst progression in the same rat model of ADPKD.

We applied sirolimus by using the Rapamune[®] oral solution. This is the galenic form which is approved to prevent graft rejection in renal transplant patients. Using a 2 mg/kg/day dose resulted in relatively low blood levels in our animals (between 0.5 and 1.9 $\mu\text{g/l}$). This indicates that the drug was absorbed poorly. Despite these low levels, the therapeutic effect was impressive. Tao *et al.* used a different preparation of sirolimus at a 10-fold lower daily dose intraperitoneally [12]. They did not measure blood levels, but judging by the significantly reduced body weight (-22%) in their rapamycin-treated rats it is likely that blood levels were higher in their animals. The 3-month treatment was well tolerated in our rats with no significant effect on body weight.

In the study by Tao *et al.*, treatment with sirolimus did not completely prevent progression of cystic disease [12]. This was the case in our study also. However, both studies show that the treatment with sirolimus markedly retarded renal functional loss. In our study we treated animals for an extended period of 3 months and found a continued inhibitory effect on renal functional loss and cystic expansion. More studies are needed to determine whether mortality is reduced in Han:SPRD rats by prolonging treatment with sirolimus or other mTOR inhibitors.

Sirolimus is known to inhibit its molecular target mTOR. As it was highly effective in Han:SPRD rats, we hypothesize that the mTOR pathway is dysregulated in the kidneys of these rats. mTOR

regulates a vast array of cellular functions, and promotes cell growth and proliferation [19]. A well-characterized function of mTOR in mammalian cells is the upregulation of protein synthesis via S6K, a direct substrate of mTOR. Phosphorylation of S6K by mTOR enhances translation of certain mRNAs, which play an important role in translation regulation, increasing thereby the overall translation capacity of cells [24].

We describe here for the first time that total amounts of S6K and activity of S6K (determined as phosphorylation of S6K) are enhanced in the kidneys of Han:SPRD rats with ADPKD and that this can be reversed by the application of sirolimus. In an acute renal failure model, it has been shown that activated S6K is increased in rat kidneys, suggesting that this signalling pathway is activated in other types of renal injury [17]. The mechanisms which lead to the enhanced expression and phosphorylation of S6K in cystic kidneys of Han:SPRD need to be investigated further. Our findings point to an important role of the mTOR pathway in Han:SPRD. We hypothesize that there could be an interaction between molecules which are defective in Han:SPRD and the mTOR pathway in the pathogenesis of cyst growth. The nature and the importance of these interactions need to be investigated in further studies.

In summary, we demonstrate that oral sirolimus markedly inhibits the rapid deterioration of renal function by inhibiting cyst growth in Han:SPRD rats with ADPKD. We speculate that mTOR and its downstream effector S6K play a critical role in the pathogenesis of ADPKD. Further studies are in progress to elucidate the mechanisms of the altered expression and phosphorylation of these proteins, and to examine the effect of mTOR inhibition in other animal models of cystic kidney disease.

Acknowledgements. This study was supported by an unrestricted research grant from Wyeth-AHP (Schweiz) AG, Switzerland (R.P.W.) and by a grant from the Swiss National Foundation (M.N.H.). Wyeth also provided Rapamune® free of charge.

Conflict of interest statement. All authors listed declare to have had no involvements that might raise the question of bias in the work or in the conclusions, implications, or opinions stated.

References

- Wilson PD. Polycystic kidney disease. *N Engl J Med* 2004; 350: 151–164
- Igarashi P, Somlo S. Genetics and pathogenesis of polycystic kidney disease. *J Am Soc Nephrol* 2002; 13: 2384–2398
- Harris PC. Molecular basis of polycystic kidney disease: PKD1, PKD2 and PKHD1. *Curr Opin Nephrol Hypertens* 2002; 11: 309–314
- The European Polycystic Kidney Disease Consortium. The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. *Cell* 1994; 77: 881–894
- The International Polycystic Kidney Disease Consortium. Polycystic kidney disease: the complete structure of the PKD1 gene and its protein. *Cell* 1995; 81: 289–298
- Mochizuki T, Wu G, Hayashi T *et al.* PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science* 1996; 272: 1339–1342
- Delmas P. Polycystins: from mechanosensation to gene regulation. *Cell* 2004; 118: 145–148
- Torres VE, Sweeney WE Jr, Wang X *et al.* Epidermal growth factor receptor tyrosine kinase inhibition is not protective in PCK rats. *Kidney Int* 2004; 66: 1766–1773
- Torres VE. Therapies to slow polycystic kidney disease. *Nephron Exp Nephrol* 2004; 98: e1–e7
- Wang X, Gattone V 2nd, Harris PC, Torres VE. Effectiveness of vasopressin V2 receptor antagonists OPC-31260 and OPC-41061 on polycystic kidney disease development in the PCK rat. *J Am Soc Nephrol* 2005; 16: 846–851
- Gretz N, Kranzlin B, Pey R *et al.* Rat models of autosomal dominant polycystic kidney disease. *Nephrol Dial Transplant* 1996; 11 [Suppl 6]: 46–51
- Tao Y, Kim J, Schrier RW, Edelstein CL. Rapamycin markedly slows disease progression in a rat model of polycystic kidney disease. *J Am Soc Nephrol* 2005; 16: 46–51
- Guba M, von Breitenbuch P, Steinbauer M *et al.* Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med* 2002; 8: 128–135
- Schmelzle T, Hall MN. TOR, a central controller of cell growth. *Cell* 2000; 103: 253–262
- Moses JW, Leon MB, Popma JJ *et al.* Sirolimus-elut stents versus standard stents in patients with stenosis in a native coronary artery. *N Engl J Med* 2003; 349: 1315–1323
- Stallone G, Schena A, Infante B *et al.* Sirolimus for Kaposi's sarcoma in renal-transplant recipients. *N Engl J Med* 2005; 352: 1317–1323
- Lieberthal W, Fuhro R, Andry CC *et al.* Rapamycin impairs recovery from acute renal failure: role of cell-cycle arrest and apoptosis of tubular cells. *Am J Physiol Renal Physiol* 2001; 281: F693–F706
- Mathieu O, Cruz-Orive LM, Hoppeler H, Weibel ER. Measuring error and sampling variation in stereology: comparison of the efficiency of various methods for planar image analysis. *J Microsc* 1981; 121: 75–88
- Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 2005; 37: 19–24
- Pearson RB, Dennis PB, Han JW *et al.* The principal target of rapamycin-induced p70S6K inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *Embo J* 1995; 14: 5279–5287
- Khaleghpour K, Pyronnet S, Gingras AC, Sonenberg N. Translational homeostasis: eukaryotic translation initiation factor 4E control of 4E-binding protein 1 and p70 S6 kinase activities. *Mol Cell Biol* 1999; 19: 4302–4310
- Isotani S, Hara K, Tokunaga C, Inoue H, Avruch J, Yonezawa K. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. *J Biol Chem* 1999; 274: 34493–34498
- Saitoh M, Pullen N, Brennan P, Cantrell D, Dennis PB, Thomas G. Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site. *J Biol Chem* 2002; 277: 20104–20112
- Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004; 18: 1926–1945

Received for publication: 3.6.05

Accepted in revised form: 6.9.05