Kidney and Blood Pressure Research

Research Article

Kidney Blood Press Res 2024;49:60–68
DOI: 10.1159/000535750

Received: July 4, 2023
Accepted: December 2, 2023
Published online: January 2, 2024

Inflammation Is More Sensitive than Cell Proliferation in Response to Rapamycin Treatment in Polycystic Kidney Disease

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Keywords
Rapamycin · Inflammation · Cell proliferation · Polycystic kidney disease

Abstract
Introduction: It has been reported that rapamycin inhibited inflammation in renal interstitial diseases. We therefore hypothesized that rapamycin could attenuate inflammation in polycystic kidney disease (PKD). ***Methods:*** Han:SPRD rats were treated with rapamycin by daily gavage from 4 weeks to 12 weeks of age at the dosage of 0.5 mg/kg/day (low dose) or 1 mg/kg/day (high dose). WT9-12 human PKD cells were treated with various concentrations of rapamycin. ***Results:*** Two-kidney/total body weight ratio and cystic index in Cy/+ kidneys were significantly reduced with the treatment of low-dose rapamycin and further reduced by the treatment with high-dose rapamycin. However, the renal function of Cy/+ rats was equally improved by the treatment with either low-dose or high-dose rapamycin. The renal cell proliferation was significantly decreased in Cy/+ kidneys with the treatment of low-dose rapamycin and was further decreased with the treatment of high-dose rapamycin as examined by Ki67 staining. The phosphorylation of S6K in cystic kidneys was decreased by low-dose rapamycin and further decreased by high-dose rapamycin. Both low-dose and high-dose rapamycin treatment decreased macrophage infiltration and the expression of complement factor B (CFB), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-alpha (TNF-α) to a similar level. The expression of CFB, MCP-1, and TNF-α and phosphorylation of S6K were inhibited in WT9-12 cells treated with 10 nM rapamycin at 24 h and 48 h, respectively. Moreover, the phosphorylation of Akt was not increased by 1 nM and 10 nM of rapamycin and enhanced by 1 μM rapamycin treatment. Interestingly, WT9-12 cell proliferation could be inhibited by 1 μM rapamycin. ***Conclusion:*** Low dose of rapamycin could inhibit inflammation and protect renal function in PKD. Inflammation is more sensitive than cell proliferation in response to rapamycin treatment in PKD.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most frequent inherited kidney disease in the world [1]. The incidence of ADPKD is 0.1–0.25%, and it accounts for 5% of patients receiving dialysis therapies [2, 3].

ADPKD is characterized by the progressive enlargement of numerous cysts in bilateral kidneys, which occupies and destroys the normal structure of the renal parenchymal, leading to end-stage renal failure in middle-aged patients [4]. The pathogenesis of ADPKD is complicated. Mutations in *PKD1* or *PKD2* gene induce aberrant cell proliferation, fluid secretion, cell-matrix interaction, cell polarity, and ciliogenesis in cyst-lining epithelial cells [5, 6]. Therapeutic strategies are designed based on these pathophysiological features of ADPKD. Targeting fluid secretion by reducing cAMP levels using tolvaptan has been approved clinically in Japan, Canada, and Europe recently [7, 8]. Exuberant inflammation is also an important feature of ADPKD [9]. It has been reported that the intermediate volume which is characterized by aberrant inflammation, fibrosis, and small cysts, is correlated with renal function decline in ADPKD patients [10]. Inhibition of inflammatory factor production or deletion of macrophages retarded cyst growth and/or prevented renal function decline in animal models of ADPKD [9, 11], which suggested that targeting inflammation may provide renal benefits for ADPKD patients.

A large array of signaling pathways are activated in ADPKD such as Ras/Raf/ERK, cAMP, mammalian target of the rapamycin (mTOR), JAK/STAT, NFAT, TGF-β, and NF-κB pathways which contribute to cell proliferation, fluid secretion, interstitial fibrosis, and inflammation in cystic kidneys [12–18]. The mTOR pathway controls protein synthesis through downstream phosphorylation of S6K and 4EBP [19]. It has been reported that mTOR is regulated by polycystin-1 and is activated in cyst-lining epithelial cells [9, 12]. Inhibition of mTOR by rapamycin (also known as sirolimus) or everolimus delayed disease progression in several orthologous and non-orthologous models of ADPKD [16, 20–25]. In contrast to the exciting results obtained from animal studies, the results in human studies are not consistent [20–22]. Early retrospective studies in kidney transplant patients with ADPKD showed that rapamycin treatment inhibited cyst growth in kidneys and livers [16, 23]. In later randomized controlled trials, rapamycin treatment did not show renal protection in ADPKD patients, although in a study with a small sample size low-dose rapamycin improved renal functions but did not inhibit kidney growth in ADPKD patients [20–22]. The low dosage of rapamycin and the feedback activation of the Akt pathway by rapamycin treatment might be the reasons for the failures in these studies [24, 21].

Rapamycin is a macrolide compound derived from the bacterium *Streptomyces hygroscopicus* [25]. It is originally used as an immunosuppressive agent in transplant patients and is used in cancer therapies later because of its anti-proliferative property [26–30]. Rapamycin also displayed anti-inflammation properties in renal interstitial diseases showing reduced production of monocyte chemoattractant protein-1 (MCP-1) and ICAM-1 and infiltration of macrophages [26, 31]. Thus, we hypothesized that rapamycin could inhibit inflammation in ADPKD. In this study, we investigated the anti-inflammatory effect of rapamycin in a non-orthologous rat model of ADPKD and an ADPKD cell culture model.

Material and Methods

Animal

The male Han:SPRD heterozygous cystic (Cy/+) and wild-type normal (+/+) rats were used, which were kept in our SPF animal facility according to the local regulations and guidelines.

Reagents

P-p70S6K (#9234S), p-4EBP1S6K (#2855P), and p-AKT(#4056) antibodies were from Cell Signaling Technology (Beverly, MA, USA). GAPDH antibody (sc-365062) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for MCP-1 (BF0678) and tumor necrosis factor-alpha (TNF-α) (BF0170) were purchased from Affinity Biosciences (Zhenjiang, China). The complement factor B (CFB) antibody (10170-1-AP) was from Proteintech (Chicago, USA). Ki67 (ab16667) antibody and CD68 antibody (ab31630) were from Abcam (Cambridge, UK). Rapamycin (MB1197-S) used for in vitro cell culture and in vivo animal experiments was purchased from Melone Pharmaceutical Co., Ltd. (Dalian, China). Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Click-iT® Edu Imaging Kits (C10340, Molecular Probes, Grand Island, NY, USA) were purchased from Invitrogen.

Experimental Animal Protocol

Male +/+ and Cy/+ rats were weaned and then treated with vehicle (0.5% sodium CMC/saline solution) (Cy/+, $n = 7$; +/+, $n = 7$), 0.5 mg/kg/day (Cy/+, $n = 7$) rapamycin, or 1.0 mg/kg/day (Cy/+, $n = 7$) rapamycin, respectively, at 4 weeks of age by gavage. Rapamycin was administered once daily for 8 weeks (day 0 to day 56). Tail blood was obtained from rats on day 56. Plasma for blood urea nitrogen (BUN) and creatinine assessment was stored at −20°C and analyzed later by kinetic color test and HPLC mass spectroscopy.

After the 8-week treatment, rats were anesthetized with chloral hydrate, and the kidneys were excised, decapsulated, and weighed. For histologic examinations, kidneys were fixed in 4% paraformaldehyde/PBS. The rest of the kidney tissue was frozen in liquid nitrogen and stored at −80°C for protein extraction.

Cell Cultures

Human immortalized ADPKD cells (WT9-12) were kindly provided by Dr. Jing Zhou (Harvard Institutes of Medicine, Harvard Medical School, Boston, MA, USA). The cells were cultured in DMEM/F-12 medium supplemented with 10% de-activated FBS. When cells reached 70–80% confluence, cells were treated with various concentrations of rapamycin for 24 h.

MTT and EdU Assay

Cell proliferation was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. WT9-12 cells were treated with various concentrations of rapamycin for 24 h. 20 μL of MTT (5 mg/mL) per well was added in a 96-well plate to each well for 4 h and then sub-cultured in the medium with 100 μL DMSO. The absorbance of each well was determined at 490 nm. The experiments were performed independently more than three times.

Cell proliferation of WT9-12 cells was further assessed by EdU assay using the Click-iT® EdU Imaging Kits. The experiments were performed according to the manufacturer's instructions. In brief, the cells were seeded in a 24-well plate and synchronized with a serum-free medium for 24 h. The wells were then treated for 24 h with various concentrations of rapamycin which was diluted in a growth medium containing 10% FBS. After 24 h rapamycin treatment, half of the media were replaced with fresh media containing 20 μM of EdU in each well for 8 h, and then the cells were fixed with 3.7% formaldehyde. For quantification of proliferation, 5 photographs were randomly taken in each well, and each field of view contained approximately 200 cells which were used to calculate the percentage of EdU-positive cells. The experiments were performed at least three times.

Histology and Immunohistochemistry Staining

Cyst volume density was assessed by morphometry, using the method of point counting as described before [32]. For morphometry, 4–6 micrographs were obtained for 1 section per rat. The pixel size of the micrographs was 1,300 × 1,030 (1.34 μm/pixel). An orthogonal grid with a line spacing of 150 pixels was used. The reviewer who performed the morphometry was blinded to experimental groups.

Immunohistochemical staining for Ki67 or CD68 was performed on 3-μm-thick tissue sections as described previously [32]. In brief, the tissue sections were deparaffinized, rehydrated, and followed by antigen retrieval. The primary antibody was incubated for 1 h, and the biotinylated secondary antibody (Vector) was incubated for 30 min on the sections. This was followed by the application of the ABC reagent (Vector). Diaminobenzidine with metal enhancement was used as the detection reagent. For proliferation index analysis, at least three sections/animals from each experimental group were assessed. Five randomly taken photos in each section were captured. Ki67-positive nuclei were counted from 400 to 500 cells (×400 magnification) in each field of view. For macrophage infiltration index, the quantification was done using on-screen grid. A grid containing 400 small squares was used to divide each photo into small areas. Each square containing the CD68 stain was scored positive. A mean of the fifteen pictures in each category was used. The Y-axis is the "percent area" of the total picture that contained CD68 cells.

Protein Extraction and Western Blot Analysis

Protein was extracted from snap-frozen kidney tissue or cultured cells as previously described [32]. Protein extracts were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Lysates in SDS-sample buffer were boiled for 5 min at 95°C, and equal protein amounts were resolved by SDS-PAGE gels before transferring to a PVDF membrane. After 1 h blocking at room temperature in blocking buffer (3% BSA in PBS with 0.05% Tween-20), the membrane was incubated for 16 h with the first antibody in 3% BSA PBS-Tween. The membrane was washed and incubated for 1 h at room temperature with a secondary antibody and then visualized by enhanced chemiluminescence detection reagents.

Statistical Analysis

Statistical analyses were performed by unpaired *t*-test or one-way ANOVA with the Newman-Keuls post hoc test using GraphPad Prism version 9.4 (GraphPad, San Diego, CA, USA). All data are expressed as means ± SD, and $p < 0.05$ was considered statistically significant.

Results

Effect of Low-Dose and High-Dose Rapamycin on Renal Function and Cyst Growth in Cy/+ Rats

The efficacy of different dosages of rapamycin was tested in the Han:SPRD rat model of ADPKD from week 4 to week 12. We found that BUN and creatinine levels were elevated in Cy/+ Han:SPRD rats at week 12 compared to those in age-matched wild-type +/+ rats. Treatment with rapamycin at the dosage of 0.5 mg/kg and 1.0 mg/kg significantly decreased the levels of BUN by 34.5% and 39.8% in Cy/+ Han:SPRD rats, respectively, compared to the controls, and there was no significant difference between these two treated groups (Fig. 1a). Similarly, the creatinine levels were also equally decreased by treatment with both dosages of rapamycin (0.5 mg/kg and 1.0 mg/kg) by 34.6% and 45.5%, respectively, in Cy/+ rats (Fig. 1a). Treatment with both dosages of rapamycin did not affect the total body weight of Cy/+ rats (Fig. 1b). However, treatment with rapamycin significantly decreased the two-kidney/total body weight ratio in Cy/+ rats by 20.4% and 37.9% in a dose-dependent manner (Fig. 1c). Similarly, the cyst volume density was also dose-dependently reduced by the treatment with 0.5 mg/kg and 1.0 mg/kg rapamycin by 30.7% and 50.9%, respectively (Fig. 1d, E). Our data suggest that low-dose and high-dose rapamycin have differential effects on renal function decline and cyst growth in Cy/+ rats.

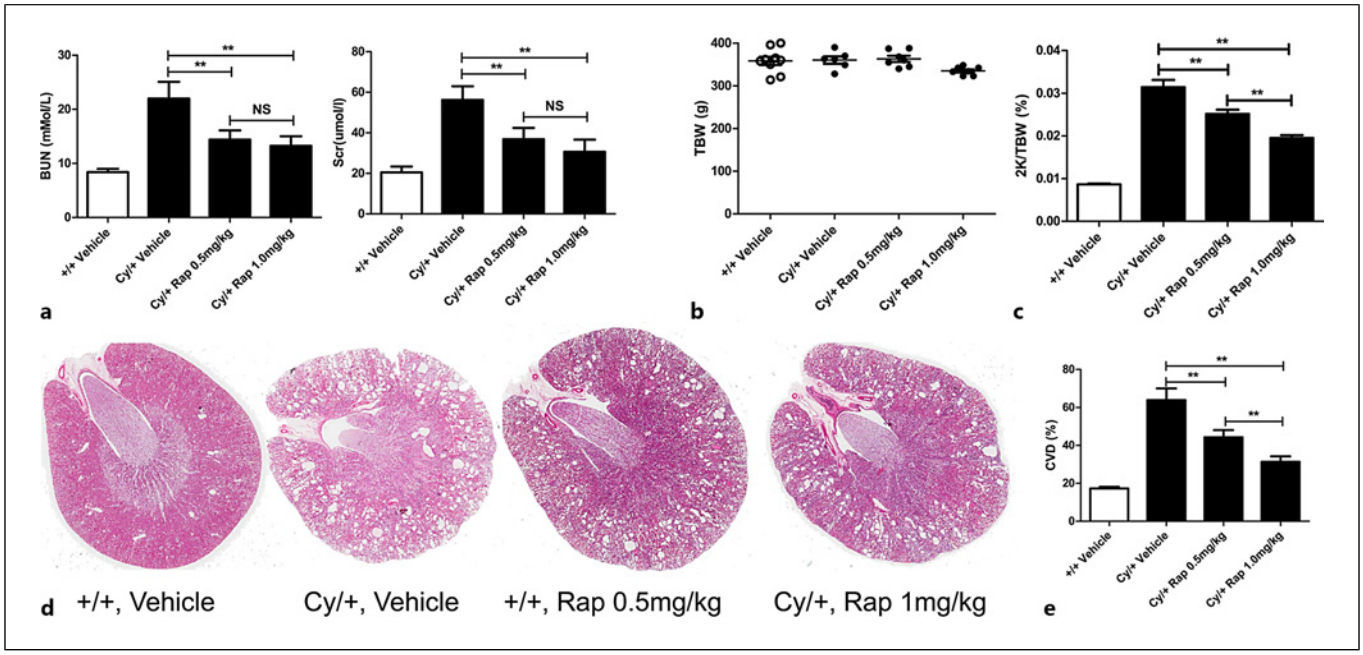

Fig. 1. Effect of rapamycin (Rap) on disease progression in a rat model of PKD. **a** BUN and creatinine levels in Rap or vehicle-treated wild type (+/+) and cystic (Cy/+) Han:SPRD rats at week 12 (7 rats in each group). **b** Total body weight (TBW) and two-kidney/total body weight (2K/TBW) ratio of 12-week-old +/+ and Cy/+ rats. **c** Hematoxylin and eosin (H&E) staining on the kidney of 12-week-old +/+ and Cy/+ rats. **d** Quantification of cyst volume density (CVD).

Effect of Low-Dose and High-Dose Rapamycin on Renal Cell Proliferation and Interstitial Macrophage Infiltration in Cy/+ Rats

We also found that treatment with a low dose and high dose of rapamycin significantly reduced the proliferation index in Cy/+ kidneys in a dose-dependent manner by 18.6% and 50.7%, respectively, as examined by Ki67 staining (Fig. 2a, b). We further found that interstitial macrophage infiltration in cystic kidneys was significantly and equally reduced with the treatment of low-dose and high-dose rapamycin by 47.8% and 56.9%, respectively, as assessed by CD68 staining. More Ki67 staining figures can be found in online supplement 1 (for all online suppl. material, see https://doi.org/10.1159/000535750).

Effect of Low-Dose and High-Dose Rapamycin on mTOR Activation and Inflammatory Factor Production in Cy/+ Rats

The activation of mTOR pathway in +/+ and Cy/+ rat kidneys was analyzed by Western blot (Fig. 3a). Phosphorylation of p70S6K, the downstream target of the mitogenic mTOR pathway, was increased in cystic kidneys and decreased by treatment with both dosages of rapamycin, whereas we observed a dose-dependent inhibition of the phosphorylation of p70S6K by rapamycin in cystic kidneys (Fig. 3a). The phosphorylation of AKT, which is a feedback target of mTOR inhibition, was increased in Cy/+ kidneys treated with rapamycin in a dose-dependent manner (Fig. 3a). In addition, the protein levels of the pro-inflammatory factor CFB, TNF-α, and MCP-1 were up-regulated in cystic kidneys from Cy/+ rats and could be equally decreased by the treatment with low-dose and high-dose rapamycin in these kidneys (Fig. 3b).

Rapamycin Inhibited ADPKD Cell Proliferation and Inflammatory Factor Production at Different Concentrations

We employed WT9-12 cells, a human ADPKD cell line, to examine the effects of rapamycin in vitro. We found that treatment with rapamycin as low as 1 μM decreased cell proliferation of WT9-12 cells as examined by MTT assay at 24 h and 48 h, respectively (Fig. 4). The inhibition of ADPKD cell proliferation by rapamycin as low as 1 μM was further confirmed by EdU assay (Fig. 4a).

We further found that treatment with rapamycin inhibited the phosphorylation of p70S6K in WT9-12 cells starting from the concentration of 1 nM at 24 h and 48 h,

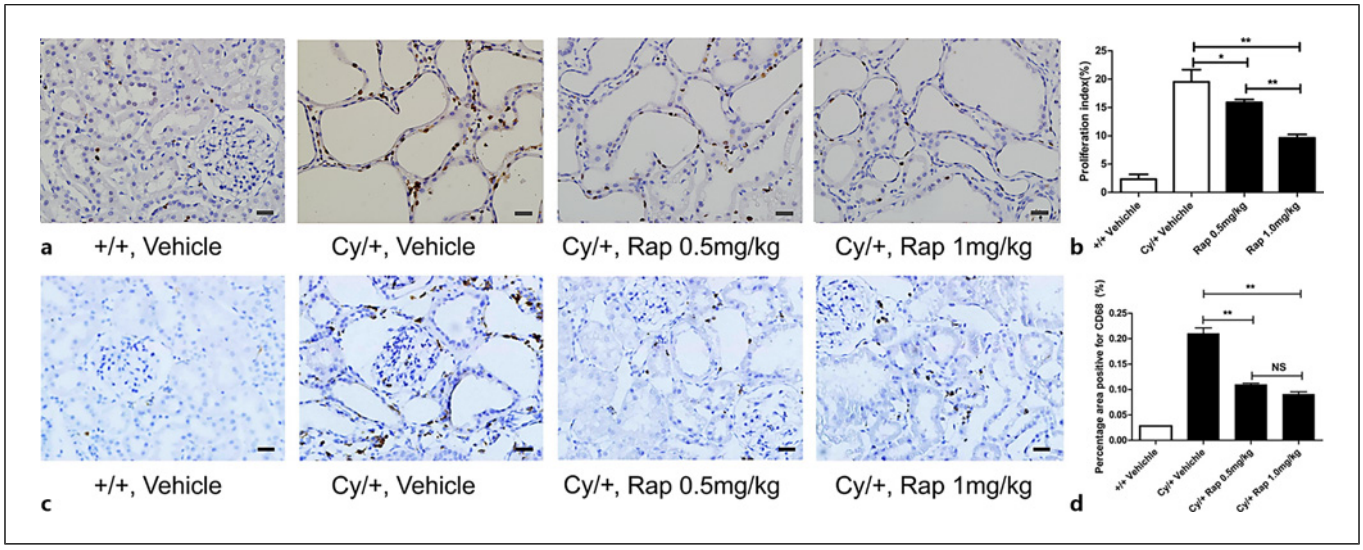

Fig. 2. Effect of rapamycin (Rap) on cell proliferation and macrophage infiltration in cystic kidneys. **a** Proliferation marker Ki67 staining was performed on 12-week-old +/+ and Cy/+ kidneys. Scale bar = 20 μm. **b** Quantification of Ki67 staining. **c** Macrophage marker CD68 staining was performed on 12-week-old +/+ and Cy/+ kidneys. Scale bar = 20 μm. **d** Quantification of CD68 staining.

respectively. Treatment with rapamycin increased the phosphorylation of AKT in the concentrations of 0.1 μM (Fig. 4c and d). However, treatment with low-dose (1 nM and 10 nM) and high-dose (10 μM) rapamycin did not increase the phosphorylation of Akt at 24 h and 48 h, respectively (Fig. 4f, g). Figure 4c and d showed that treatment with rapamycin dose-dependently decreased the expression of CFB and TNF-α in WT9-12 cells starting from 10 nM at 24 h and 48 h, respectively. Furthermore, rapamycin treatment significantly decreased the secretion of MCP-1 into the supernatant of the rapamycin-treated cells, even at 1 nM, compared to that in the control cells, as determined with ELISA (Fig. 4e).

Discussion

In this study, we showed that low-dose rapamycin had a similar effect on renal function improvement as high-dose rapamycin did, whereas treatment with high-dose rapamycin was more effective in delaying cyst growth than low-dose rapamycin in a non-orthologous rat model of ADPKD. Our study is consistent with a previous study by Novalic et al. [24] performed in a *Pkd1* mouse model, in which low-dose rapamycin improved renal function but did not inhibit kidney growth in ADPKD mice. The intriguing findings in our study were that rapamycin also inhibited interstitial inflammation in PKD, in which treatment with low-dose rapamycin had a similar effect on inflammation inhibition as high-dose rapamycin did, and inflammation was more sensitive than cell proliferation in response to rapamycin treatment.

Although ADPKD has always been thought to be predominantly a hereditary ailment, new research indicates that the kidney's immunological microenvironment may have a significant impact on the course and severity of the disease [9, 11, 31, 38]. Inflammation is an important feature of ADPKD. Inhibition of inflammation improved renal function and/or retarded kidney growth in different models of PKD [9, 11, 31, 38]. Caroli et al. [10] reported that the intermediate volume, which was characterized by aberrant inflammation and interstitial fibrosis instead of kidney size, was tightly correlated with renal function decline in ADPKD patients. Our animal study supported this notion by showing that inhibition of inflammation by rapamycin was tightly correlated with renal function improvement.

Rapamycin was originally used as an immunesuppressant agent in kidney transplantation, whereas it was mainly considered an anti-proliferative drug in later studies for cancer therapies [26–30]. Several studies showed that rapamycin improved renal interstitial disease by inhibiting inflammation [26, 31]. In this study, we found that rapamycin inhibited inflammation in PKD in vivo and in vitro. Importantly, we found that

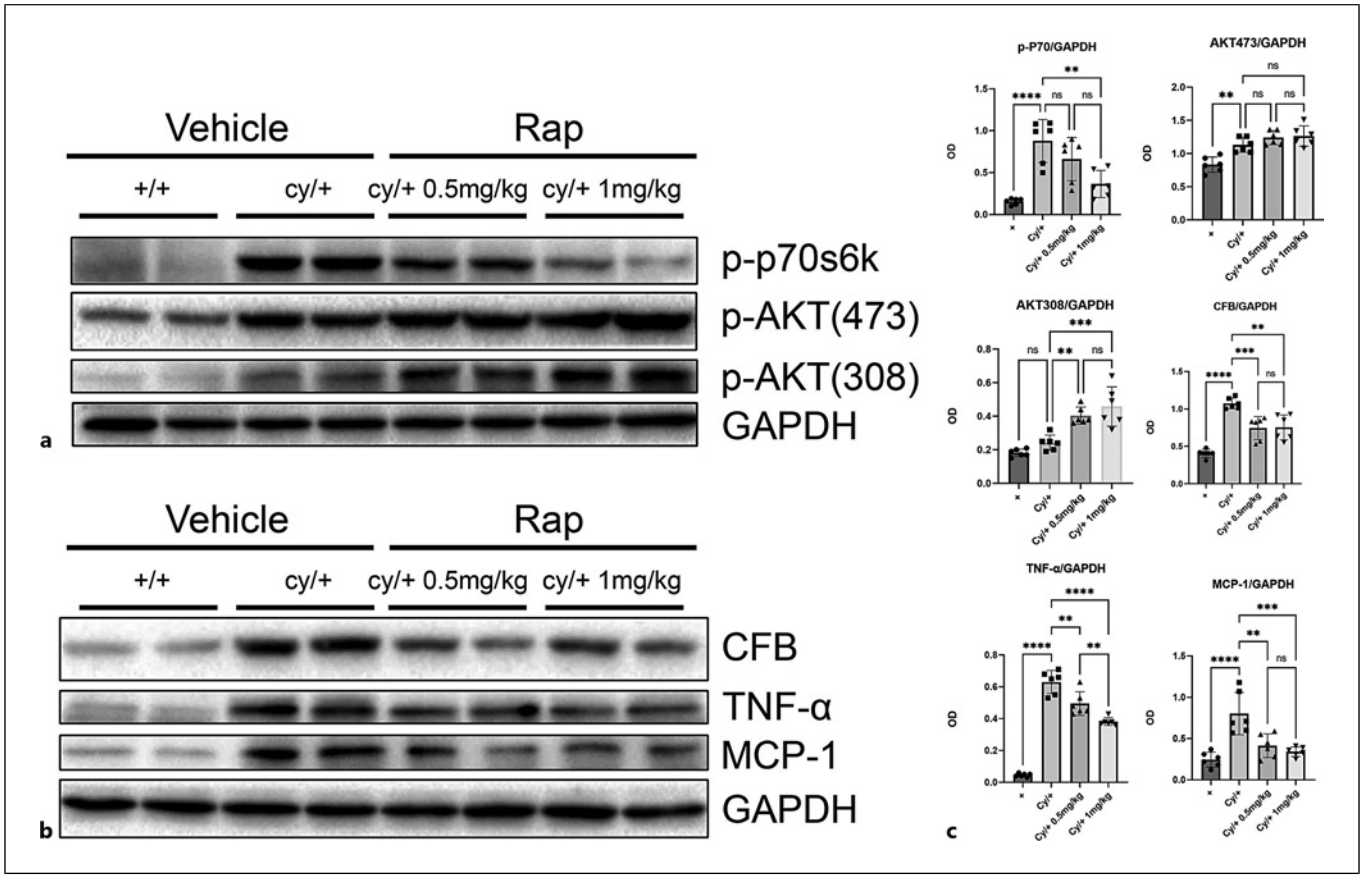

Fig. 3. mTOR cell signaling pathways and pro-inflammatory factors in rapamycin (Rap) or vehicle-treated cystic kidneys. **a** The inhibition of the mTOR pathway (p-p70S6K) and the feedback activation of Akt (p-Akt) were analyzed by Western blot in 12-week-old +/+ and Cy/+ kidneys (6 rats in each group). **b** The expression of CFB, TNF-α, and MCP-1 was analyzed by Western blot in 12-week-old +/+ and Cy/+ kidneys. Blots are representative of three independent experiments. **c** Western blot expression quantified by densitometry, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

inflammation is more sensitive than cell proliferation in response to rapamycin treatment in PKD. In rat cystic kidneys, macrophage infiltrations and inflammatory factor productions were equally inhibited by low-dose and high-dose rapamycin. However, cell proliferation was less inhibited by low-dose rapamycin than by high-dose rapamycin in PKD rats. In ADPKD cells, the concentration of rapamycin used to inhibit inflammation is at least two orders of magnitude lower than that used to inhibit cell proliferation. This result suggests that a lower concentration of rapamycin can be used just as an anti-inflammatory agent for this lifelong disease to only prevent renal function decline.

The dosage of rapamycin used in ADPKD studies was 4–10 μmol/L, and it will be cytotoxic if the blood concentration of rapamycin is over 10 μmol/L [21]. According to our in vitro study, treatment with 1 μM of rapamycin already exerted its anti-proliferative property on ADPKD cells, whereas a higher concentration of rapamycin was required to be delivered into cystic kidneys to inhibit cystic renal epithelial cell proliferation in ADPKD patients. Our results may explain the outcome in a randomized study with 30 ADPKD patients, in which 12-month treatment with a low concentration of rapamycin (trough level, 2–5 ng/mL) improved renal functions but did not change kidney size compared with standard care group or standard-dose rapamycin group (trough level, 5–8 ng/mL) [33].

Feedback activation of the mitogenic Akt pathway by rapamycin treatment is an important concern for clinicians to treat ADPKD patients with rapamycin [34]. We did observe a feedback activation of Akt in vivo and in vitro after rapamycin treatment; however, the increased phosphorylation of Akt did not result in an increase in cell proliferation in cystic kidneys and ADPKD

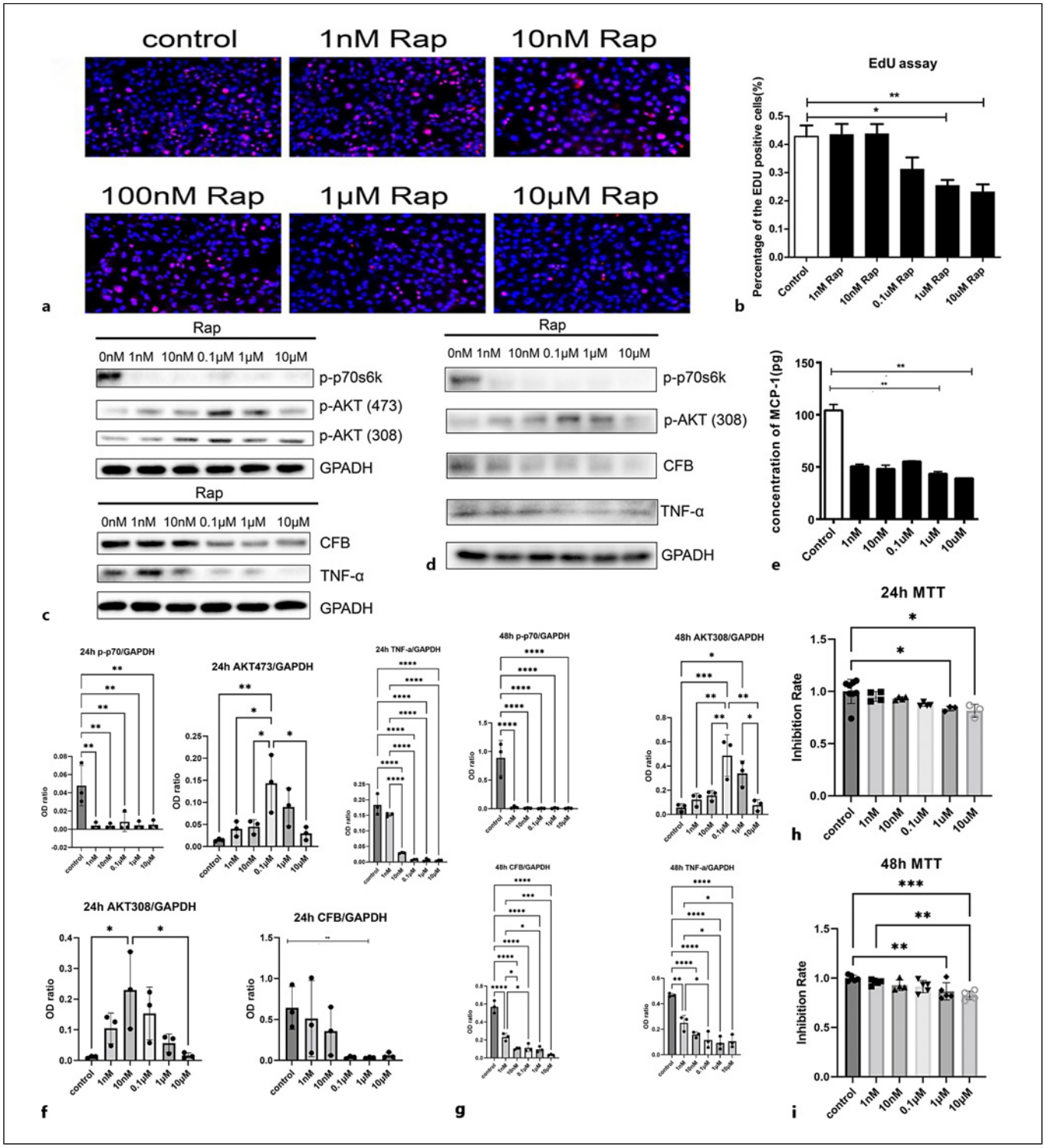

Fig. 4. Effect of rapamycin (Rap) in ADPKD cells. **a**, **b** ADPKD cells (WT9-12) were exposed to 1 nM, 10 nM, 0.1 μM, 1 μM, and 10 μM Rap for 24 h. Cell proliferation was evaluated using the (**a**, **b**) EdU assay, and the percent of the positive area was quantified. One representative result of three independent experiments was shown. **c**, **d** The inhibition of the mTOR pathway (p-p70S6K) and the feedback activation of Akt (p-Akt), CFB, and TNF-α were analyzed by Western blot in Rap-treated ADPKD cells at 24 h (**c**) and 48 h (**d**), respectively. Blots represented three independent experiments. **e** Supernatant MCP-1 of Rap-treated ADPKD cells was measured by ELISA. One representative result of three independent experiments was shown. **f**, **g** Western blot expression of 24 h (**c**) and 48 h (**d**) were quantified by densitometry, respectively. **h**, **i** MTT assay of 24 h (**h**) and 48 h (**i**). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

cells. Since inhibition of inflammation could also attenuate cell proliferation in PKD, the feedback activation of the mitogenic Akt pathway was probably balanced by the inhibition of inflammation by rapamycin treatment [9, 11]. It has been reported that prolonged rapamycin treatment could inhibit Akt activation [35]. In the present study, we observed that the feedback activation of Akt was not activated by low-dose (1 nM and 10 nM) rapamycin and attenuated by a higher concentration of rapamycin. Therefore, a relatively low concentration of rapamycin treatment could be used to inhibit inflammation without activating the proliferative feedback in ADPKD. Otherwise, a higher local concentration of rapamycin treatment also could be used to reduce cystic cell proliferation by inhibiting the Akt pathway, if the cytotoxic effect of rapamycin can be resolved.

Taken together, our study indicated that low-dose rapamycin could inhibit inflammation and protect renal function in PKD. Low-dose rapamycin may serve as a sensitive inflammation inhibitor rather than a cell proliferation inhibitor for PKD therapy in the future.

Acknowledgment

We thank Dr. Ming Wu for his guidance in this manuscript.

Statement of Ethics

The study was approved by the Ethics Review Board of Changzheng Hospital (CZ2021-0616).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by the National Natural Science Foundation of China (81770706 and 82200786), grants from Changzheng Hospital Excellent Young Physician Program to Lv, the project of the Special Fund for Scientific Research Start-Up of Shanghai Fourth People's Hospital (No. sykyqd07601), and the Program of Shanghai Academic/Technology Research Leader (No. 23XD1402600).

Author Contributions

Concept or design, acquisition of data, and analysis or interpretation of data: Ming Yang, Jiayi Lv, Chanjuan Gong, Cheng Xue, Lili Fu, and Changlin Mei. Drafting of the manuscript: Ming Yang, Cheng Xue, Jiayi Lv, Chanjuan Gong, Shunjie Chen, and Changlin Mei. Critical revision of the manuscript for important intellectual content: all authors. All authors had full access to the data, contributed to the study, approved the final version for publication, and took responsibility for its accuracy and integrity.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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