Transpl Int (1994) 7: 83–88

ORIGINAL ARTICLE

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Renal effects of rapamycin in the spontaneously hypertensive rat

Received: 30 December 1992
Received after revision: 2 June 1993
Accepted: 4 June 1993

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Abstract The effects of rapamycin (RAPA), administered at therapeutic doses, were investigated in the spontaneously hypertensive rat (SHR). Additionally, the reversibility of RAPA's renal effects was investigated at a supratherapeutic dose. At doses that were active in preventing heart and kidney allograft rejection in the rat (0.01–0.08 mg/kg i.v.), RAPA had no effect on kidney function or rat body weight gain. At higher doses (0.8 mg/kg), RAPA produced significant changes in kidney function parameters and caused a loss in body weight. Histopathologic changes, including necrotizing vasculopathy and tubular atrophy, were noted at therapeutic doses. The effects of RAPA on kidney function were completely reversible after a 2-week washout period, though the histopathologic changes were still evident. These studies demonstrate that RAPA does not impair kidney function at therapeutic doses when administered for 2 weeks but does appear to accelerate the naturally occurring renal lesions of the SHR.

Key words Rapamycin, rat, renal function · Renal function, rapamycin, rat · Spontaneously hypertensive rat, rapamycin

Introduction

Rapamycin (RAPA) has demonstrated potent immunosuppressive activity in transplant models in the mouse, rat, dog, pig, and primate [13, 14]. RAPA exerts its immunosuppressive effects primarily by suppressing T-cell proliferation but through a mechanism distinct from both cyclosporin A (CyA) and FK506. CyA and FK506 block the Ca^{++} and calmodulin-dependent phosphatase calcineurin, resulting in the inhibition of IL-2 transcription [17]. RAPA has no effect on these events. It inhibits T-cell proliferation by blocking IL-2-mediated signal transduction pathways [2]. Although the molecular targets of RAPA's effects have not been identified unequivocally, it has recently been reported that RAPA inhibits p70 S6 kinase activation by preventing its phosphorylation [4]. The very distinct differences in the mechanism of T-cell inhibition by RAPA versus CyA and FK506 may result in a different, and possibly better, side effect profile for RAPA.

The most limiting side effect of CyA is nephrotoxicity, which also hampers the clinical use of FK506 [1]. RAPA's effect on renal function has been characterized in preclinical models, particularly the mouse [5, 11] and rat [6, 8, 9, 21–23]. RAPA had little or no effect on renal function and histopathology in the mouse [5, 11], Sprague-Dawley rat [8, 9, 21, 22], Lewis rat [9, 23], and female spontaneously hypertensive rat (SHR) [6]. In the male SHR, RAPA caused renal dysfunction and histopathologic alterations, but at doses 25–100 times higher than the therapeutic doses established for RAPA [9]. The SHR strain is unique in that it spontaneously produces renal lesions consisting of necrotizing vasculopathy and tubular atrophy with the development of hypertension. Since the previous studies were conducted with supratherapeutic doses of RAPA, this current study analyzed the effects of RAPA at therapeutic doses on renal function and histopathology in this nephrosensitive strain of rat, the SHR. The use of osmotic minipumps to administer RAPA in this study was identi-

Table 1 Kidney function in the spontaneously hypertensive rat. Continuous i. v. infusion of low doses of rapamycin (RAPA) for 14 days (mean ± SEM). * $P < 0.05$ vs vehicle

Treatment (mg/kg i.v.)	n	Δ Body weight[a] (g)	Urine volume (ml/17 h)	Plasma creatinine (mg/dl)	Creatinine clearance (ml/min per 100 g)
Vehicle	7	+39.4	13.3 ± 1.7	0.27 ± 0.02	0.76 ± 0.04
RAPA 0.01	7	+34.4	15.8 ± 2.9	0.21 ± 0.02	0.82 ± 0.07
RAPA 0.02	7	+27.1	17.4 ± 1.4	0.20 ± 0.02*	0.87 ± 0.1
RAPA 0.04	6	+26.2	17.4 ± 0.9	0.17 ± 0.02*	1.05 ± 0.11

[a] Body weight = day 14–day 0

cal to the means of drug administration used to determine the therapeutic doses for RAPA in rat allograft models [3, 19, 20].

Materials and methods

Male SHR (initial weight range 191–233 g) were obtained from Charles River (Wilmington, Ma., USA). The animals were allowed free access to food and water throughout the study.

RAPA was solubilized in IV-5 (20% N,N-dimethyl acetamide, 10% Tween 80, and 70% polyethylene glycol 400). CyA was solubilized in 35% ethanol and 65% cremophor. Drug or vehicle was loaded into Alzet (Palo Alto, Calif., USA) osmotic minipumps (models 2002, flow rate 0.5 µl/h or model 2 ml-2, flow rate 5.0 µl/h) for continuous intravenous administration for 2 weeks. RAPA stability and its delivery by osmotic pump has been confirmed by HPLC [10]. The concentration of RAPA or CyA was adjusted to deliver a dose based on day 0 average rat weights. Pumps were allowed to equilibrate overnight at 37 °C in sterile saline before implantation.

Pump implantation

Rats were anesthetized with Nembutal (pentobarbital, 20 mg/rat i.p.). The jugular vein was cannulated with PE50 tubing. The tubing was tunneled under the skin and connected to the osmotic pump, which was placed subcutaneously in the back of the rat. Incisions were closed with wound clips and the rats were allowed to recover from anesthesia.

Protocol

On day 14 of each study, rats were placed into individual metabolism cages and urine was collected for 17 h. The urine was centrifuged (at 800 *g* for 10 min) to remove solid particles and then frozen (− 20°C). For the reversibility study, kidney function for half of the treatment group was analyzed on day 15 of the study while the remaining half was analyzed on day 29 after overnight urine collection. For each of the studies, Fluothane-anesthetized rats were bled by cardiac puncture into heparinized tubes. Plasma was collected after centrifugation (at 1500 *g* for 30 min) and frozen (− 20°C). The rats were euthanized by an overdose of Fluothane and the kidneys were collected, weighed, and then placed into 10% buffered formalin for histologic analysis. Kidney weights were adjusted for rat body weights.

Biochemical determinations

Levels of urine and plasma creatinine were determined with an Abbott VP Biochromatic Analyzer by a colorimetric reaction using alkaline picrate as reagent. Creatinine clearance (CrCL) was determined with the following equation: Clearance rate

$$(\text{ml/min}) = \frac{\text{Urine concentration}}{\text{Plasma concentration}} \times \text{Urine flow rate (ml/min)}$$

Clearance values were expressed per 100 g of body weights.

Histology

Kidneys were embedded in paraffin, sectioned at 3 µ, and stained with hematoxylin and eosin, periodic acid-Schiff, periodic acid-Schiff with silver, and chromotrope aniline blue and scored for the following: proximal tubular epithelium inclusion bodies, necrotizing vasculopathy, tubular atrophy, proximal tubular epithelium vacuolization, microcalcification, and hypertrophy of the juxtaglomerular apparatus; a grade of 4 was considered severe. Light microscopic histopathologic examination of the kidney sections was performed in a blinded fashion by one of us (MJM). The incidence and severity of lesions observed in each treatment group was recorded. For the reversibility study, kidneys were stained with hematoxylin and eosin and light microscopic examination was performed by R. Sharma, Ph. D., consulting pathologist, in a blinded fashion.

Statistics

Results are presented as treatment group means ± SEM. Percentage change from the vehicle-treated group was calculated. Statistical significance versus vehicle-treated rats was determined by Dunnett's multiple comparison technique using a 95% confidence level.

Results

Dose response studies

Three independent studies were conducted using overlapping doses of RAPA. In the low-dose study, RAPA (0.01–0.04 mg/kg) had no significant effect on body weight gain, urine output, or CrCL (Table 1). Plasma creatinine values were significantly decreased at both 0.02 and 0.04 mg/kg in this initial study. In the second study, where intermediate doses were used (0.02–0.08 mg/kg), RAPA again had

Table 2 Kidney function in the spontaneously hypertensive rat. Continuous i. v. infusion of intermediate doses of rapamycin (RAPA) for 14 days (mean ± SEM). * $P < 0.05$ vs vehicle

[a] Body weight = day 14–day 0

Treatment (mg/kg i. v.)	*n*	Δ Body weight[a] (g)	Urine volume (ml/17 h)	Plasma creatinine (mg/dl)	Creatinine clearance (ml/min per 100 g)
Vehicle	6	+ 28.2	12.0 ± 1.1	0.55 ± 0.01	0.30 ± 0.01
RAPA 0.02	7	+ 32.7	13.7 ± 0.7	0.51 ± 0.02	0.33 ± 0.01
RAPA 0.04	7	+ 35.3	15.5 ± 1.6	0.48 ± 0.02*	0.39 ± 0.04
RAPA 0.08	7	+ 23.8	17.4 ± 2.6	0.55 ± 0.02	0.31 ± 0.05

Table 3 Kidney function in the spontaneously hypertensive rat. Continuous i. v. infusion of high doses of rapamycin (RAPA) for 14 days (mean ± SEM). * $P < 0.05$ vs vehicle

[a] Body weight = day 14–day 0

Treatment (mg/kg i. v.)	*n*	Δ Body weight[a] (g)	Urine volume (ml/17 h)	Plasma creatinine (mg/dl)	Creatinine clearance (ml/min per 100 g)
Vehicle	7	+ 49.2	15.8 ± 1.1	0.50 ± 0.02	0.42 ± 0.04
RAPA 0.08	7	+ 22.5*	18.5 ± 3.3	0.55 ± 0.01	0.35 ± 0.03
RAPA 0.8	5	− 13.2*	14.6 ± 3.3	0.58 ± 0.02*	0.31 ± 0.01*

Table 4 Kidney function in the spontaneously hypertensive rat. Continuous i. v. infusions of cyclosporin A (CyA) for 14 days (mean ± SEM). * $P < 0.05$ vs vehicle

[a] Body weight = day 14–day 0

Treatment (mg/kg i. v.)	*n*	Δ Body weight[a] (g)	Urine volume (ml/17 h)	Plasma creatinine (mg/dl)	Creatinine clearance (ml/min per 100 g)
Vehicle	7	+ 34.6	13.3 ± 0.9	0.39 ± 0.02	0.47 ± 0.08
CyA 2.0	7	+ 16.7*	14.7 ± 1.4	0.42 ± 0.02	0.52 ± 0.08
CyA 5.0	7	+ 9*	11.8 ± 1.9	0.4 ± 0.02	0.39 ± 0.04

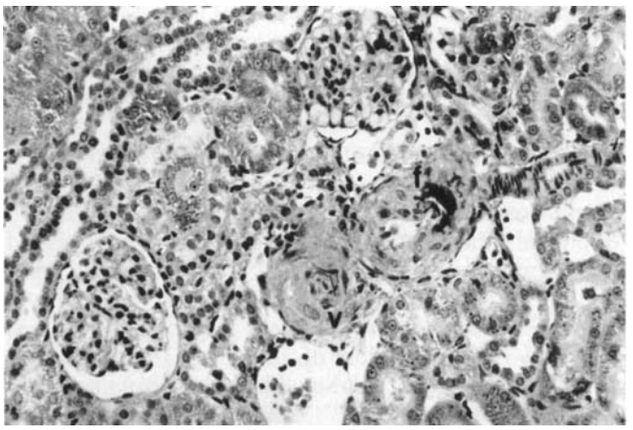

Fig. 1 Rapamycin (0.08 mg/kg i. v. for 14 days)-treated spontaneously hypertensive rat kidney demonstrating necrotizing vasculopathy (*v*) with fibrinoid necrosis, represented by the large black area (*f*) surrounded by slightly atrophic tubules. Glomeruli appear normal (× 280)

no significant effect on body weight gain or any parameter of kidney function except for a decrease in plasma creatinine observed only at the 0.04 mg/kg dose (Table 2). In the high-dose study, rats treated with 0.08 mg/kg RAPA gained body weight but at a rate significantly less than vehicle-treated rats. At 0.08 mg/kg, RAPA did not significantly effect any parameter of kidney function (Table 3). For the highest dose (0.8 mg/kg), there was a significant decrease in rat body weight over the 14-day test period. Plasma creatinine was significantly elevated by 16 % while CrCL was significantly depressed. RAPA had no effect on kidney weights in any of the studies. The vehicle (IV-5) had no effect on any kidney function parameter when compared with saline-treated rats (data not presented).

CyA, given at 2 mg/kg by continuous intravenous infusion for 2 weeks, had no significant effect on any parameter of kidney function but did cause a significant retardation in body weight gain (Table 4). When given at 5 mg/kg, CyA caused a larger decrease in body weight gain and a 20 % decrease in creatinine clearance, which did not achieve statistical significance. The cremophor-ethanol formulation used to solubilize and deliver CyA did not have any effect on kidney function (unpublished results).

Histopathology

In all three studies, RAPA increased the frequency and severity of necrotizing vasculopathy (Fig. 1). The incidence ranged from 57 % to 100 % with a severity score of mild to moderate (Table 5). The effects did not appear to be dose-dependent. RAPA treatment increased the in-

Table 5 Summary table; histopathologic analysis of rapamycin-treated spontaneously hypertensive rat kidneys. IB, Inclusion bodies of the proximal tubule; VASC, necrotizing vasculopathy; TUB, tubular atrophy

Treatment (mg/kg i.v.)	% Incidence (average severity)			
	n	IB	VASC	TUB
Vehicle	20	10 (1.0)	0	0
RAPA 0.01	7	14 (1.0)	57 (1.5)	0
RAPA 0.02	14	43 (1.2)	93 (1.5)	7 (2.0)
RAPA 0.04	13	23 (1.3)	92 (2.1)	15 (1.0)
RAPA 0.08	14	50 (1.4)	93 (1.7)	29 (2.3)
RAPA 0.8	5	100 (1.2)	60 (1.3)	100 (2.0)

cidence and severity of tubular atrophy; at the high dose of 0.8 mg/kg, 100% incidence was noted with a severity grade of moderate. Tubular vacuolization was only noted in one rat at the dose of 0.02 mg/kg, while no evidence of drug-induced inclusion bodies, microcalcification, or juxtaglomerular hypertrophy was noted in RAPA-treated rats relative to vehicle controls. The tubular lesions (inclusion bodies and vacuolization) observed in this study were minimal and not different from those seen in vehicle-treated controls.

CyA caused a dose-dependent increase in the incidence and severity of vasculopathy. At the lower dose of 2 mg/kg, CyA produced a 29% incidence of vasculopathy with an average severity of 1.0 (mild) and, at 5 mg/kg, produced a 57% incidence with a severity of 1.8 (mild). The incidence of tubular atrophy went from 43% at the lower dose to 71% at the higher dose, both rated as mild to moderate in severity. Tubular vacuolization was observed in both treatment groups. The incidence was not dose-dependent and was graded as mild in severity. Inclusion bodies were observed but the incidence and severity were not different from those in the vehicle-treated rats.

Reversibility study

Two groups of rats were implanted with osmotic pumps for a 14-day infusion of RAPA, 1.0 mg/kg, or vehicle, IV-5. Half of the rats (RAPA $n = 6$, vehicle $n = 6$) were assessed on day 15 for kidney function and then sacrificed while the remaining rats (RAPA $n = 7$, vehicle $n = 6$) were assessed on day 29 and then sacrificed. The second set of rats had been without drug treatment for 14 days (washout period). Vehicle-treated rats gained 36.5 g over the first 14 days and 40 g over the next 14 days (Table 6). During drug infusion, RAPA-treated rats gained 9.1 g over the first 2 weeks and 28.8 g during the washout period. Even though the RAPA-treated weight gain was significantly less than that with the vehicle for both time periods, the washout period weight gain was approaching normal vehicle-treated values. RAPA-treated rats, on day 15, had a small increase in plasma creatinine values and a significant decrease in CrCL. After the washout period, the plasma creatinine values were significantly lower than the vehicle-treated ones, and CrCL values had returned to normal vehicle-treated levels, although urine output was significantly increased. Therefore, the kidney dysfunction and weight loss observed after 2 weeks of treatment with a supratherapeutic dose of RAPA was reversible after a 2-week washout period.

The histopathologic changes induced by the 2-week RAPA treatment were not reversible by the washout period. After 2 weeks of drug infusion, RAPA-treated kidneys exhibited vasculopathy (moderate severity) that was not observed in vehicle-treated rats. After the washout period, a higher incidence of vasculopathy (43%) and tubular atrophy (43%), both of moderate severity, was noted relative to both vehicle-treated kidneys and RAPA-treated kidneys analyzed at day 14.

Discussion

RAPA had no detrimental effects on kidney function or body weights at doses of 0.08 mg/kg and lower in the SHR. These doses were active in heart, kidney, and pancreoduodenal transplant models in rats where RAPA was administered by osmotic pump [19, 20], identical to the method of drug administration employed in the present study. Small bowel and pancreas transplants were protected by RAPA at a dose of 0.8 mg/kg [3, 19, 20]. Lower doses were not reported. Small bowel transplantation in-

Table 6 Reversibility of rapamycin's effect on kidney function in the spontaneously hypertensive rat. * $P < 0.05$ vs vehicle

Treatment	*n*	Δ Body weight (g)	Urine volume (ml/17 h)	Plasma creatinine (mg/dl)	Creatinine clearance (ml/min per 100 g)
2-week infusion (Day 14)					
Vehicle	6	+36.5[a]	9.0 ± 0.8	0.47 ± 0.02	0.53 ± 0.03
RAPA (1 mg/kg i.v.)	6	+ 9.1*	7.9 ± 1.1	0.52 ± 0.02	0.37 ± 0.06*
2-week washout (Day 28)					
Vehicle	6	+40.0[b]	7.8 ± 0.5	0.51 ± 0.01	0.47 ± 0.02
RAPA	7	+28.8*	13.7 ± 2.4*	0.45 ± 0.01*	0.52 ± 0.02

[a] Body weight = day 14–day 0 weight
[b] Body weight = day 28–day 14 weight

duces both allograft rejection and graft-versus-host disease, so a drug that is active in this model is a highly effective immunosuppressant. At 0.8 mg/kg, kidney dysfunction and body weight loss were observed in the SHR.

Histopathologic examination of SHR kidneys revealed the presence of necrotizing vasculopathy, even at the lowest doses of 0.01 mg/kg. The effects were not dose-dependent. Tubular atrophy was observed in an inconsistent manner throughout the three studies. RAPA did not induce tubular inclusion bodies of greater severity than in vehicle-treated rats, nor did it produce microcalcifications or juxtaglomerular hypertrophy. Tubular vacuolization, inclusion bodies, and microcalcifications have been observed in CyA-treated SHRs [9, 15]. In the current study, tubular vacuolizations, atrophy, and necrotizing vasculopathy were observed.

The reversibility study using a supratherapeutic dose of 1 mg/kg demonstrated that complete normalization of kidney function was obtained after a 2-week washout period for RAPA. At this time point, the histopathologic changes were not reversed. For CyA, a 10-day washout period was not able to reverse the histopathologic changes present after a 10-day treatment period [15].

Based on comparative data with other strains of rats [9], the SHR seems to be the most sensitive to RAPA's renal effects. This enhanced sensitivity may be due to the strain's predisposition to develop necrotizing vasculopathy and tubular atrophy with age [12], the two lesions observed in RAPA-treated SHRs. The spontaneous development of renal lesions has been dissociated from the hypertension that also occurs in the model [12, 16]. Preliminary results indicate that RAPA does not produce hypertension in the SHR [7]. RAPA, therefore, seems to accelerate the natural occurrence of renal lesions in the SHR and this effect is independent of RAPA's blood pressure effects.

A dose of 2 mg/kg of CyA (administered intravenously by constant infusion by an osmotic pump for 2 weeks) did not significantly protect against heart allograft rejection [18] but did protect pancreoduodenal allografts [3]. At this dose of CyA, no effects on kidney function were observed in the SHR, but body weight loss and histopathologic changes, including vasculopathy, tubular atrophy, and tubular vacuolization, were observed. CyA, therefore, at doses that do not significantly protect against graft rejection in heart allografts still produced body weight loss and kidney histopathology. RAPA, at immunosuppressive doses of 0.08 mg/kg and lower, had no effect on body weights or kidney function, nor did it produce tubular lesions or microcalcifications.

RAPA, therefore, does not effect kidney function in the SHR at therapeutic doses. Even though RAPA did produce histopathologic changes, the profile of its changes was different from that reported for CyA. RAPA primarily accelerates the spontaneously occurring lesions in the SHR (necrotizing vasculopathy and tubular atrophy), while CyA causes tubular lesions and also accelerates the appearance of the spontaneous lesions. These observations support the contention that the distinct mechanism of immunosuppression between RAPA and CyA will result in a different profile of side effects. In preclinical studies investigating renal function, RAPA has a better separation between therapeutic doses and toxic doses than CyA.

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