BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 43 (8) 698-811 August 2010

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, August 2010, Volume 43(8) 737-744

doi: 10.1590/S0100-879X2010007500058

Effects of sirolimus alone or in combination with cyclosporine A on renal ischemia/reperfusion injury

B.J. Pereira, I. Castro, E.A. Burdmann, D.M.A. Malheiros and L. Yu

Brazilian Journal of Medical and Biological Research (2010) 43: 737-744
ISSN 0100-879X

Effects of sirolimus alone or in combination with cyclosporine A on renal ischemia/reperfusion injury

B.J. Pereira, I. Castro, E.A. Burdmann, D.M.A. Malheiros and L. Yu

Divisão de Nefrologia (LIM 12), Faculdade de Medicina,
Universidade de São Paulo, São Paulo, SP, Brasil

Abstract

Calcineurin inhibitors exacerbate ischemic injury in transplanted kidneys, but it is not known if sirolimus protects or exacerbates the transplanted kidney from ischemic injury. We determined the effects of sirolimus alone or in combination with cyclosporin A (CsA) on oxygenated and hypoxic/reoxygenated rat proximal tubules in the following *in vitro* groups containing 6-9 rats per group: sirolimus (10, 50, 100, 250, 500, and 1000 ηg/mL); CsA (100 µg/mL); sirolimus (50 and 250 ηg/mL) + CsA (100 µg/mL); control; vehicle (20% ethanol). For *in vivo* studies, 3-week-old Wistar rats (150-250 g) were submitted to left nephrectomy and 30-min renal artery clamping. Renal function and histological evaluation were performed 24 h and 7 days after ischemia (I) in five groups: sham, I, I + SRL (3 mg·kg^{-1}·day^{-1}, *po*), I + CsA (3 mg·kg^{-1}·day^{-1}, *sc*), I + SRL + CsA. Sirolimus did not injure oxygenated or hypoxic/reoxygenated proximal tubules and did not potentiate the tubular toxic effects of CsA. Neither drug affected the glomerular filtration rate (GFR) at 24 h. GFR was reduced in CsA-treated rats on day 7 (0.5 ± 0.1 mL/min) but not in rats receiving sirolimus + CsA (0.8 ± 0.1 mL/min) despite the reduction in renal blood flow (3.9 ± 0.5 mL/min). Acute tubular necrosis regeneration was similar for all groups. Sirolimus alone was not toxic and did not enhance hypoxia/reoxygenation injury or CsA toxicity to proximal tubules. Despite its hemodynamic effects, sirolimus protected post-ischemic kidneys against CsA toxicity.

Key words: Cyclosporin A; Nephrotoxicity; Renal proximal tubules; Renal ischemia

Introduction

Cyclosporin A (CsA) is one of the mainstays of immunosuppression in solid organ transplantation, having provided significant improvement in the clinical outcome of organ transplantation. CsA is a calcineurin phosphatase inhibitor that prevents the transcription of cytokines and the progression of the T-cell cycle from G0 to G1 (1). Despite the pivotal role played by CsA in clinical transplantation, drug-induced nephrotoxicity remains a serious clinical limitation. Abnormalities in renal hemodynamics, especially efferent arteriolar vasoconstriction, constitute the main mechanism of acute CsA nephrotoxicity. In addition, long-term CsA administration can lead to chronic nephropathy, characterized by local inflammation and irreversible interstitial fibrosis (2-4). In addition to experimental drug-induced changes in renal hemodynamics, CsA can also cause direct tubular injury (5). Sirolimus (SRL) is an immunosuppressant used for the prevention and treatment of renal transplant rejection. Rather than working through the calcineurin pathway (1), SRL inhibits the activity of P70 S6 kinase, affecting critical biochemical events later in the T-cell cycle, preventing the progression from the G1 phase to the S phase. In addition, SRL blocks the T-cell proliferation induced by cytokines, alloantigens and mitogens. SRL is rapidly absorbed, well-tolerated and safe. In contrast to calcineurin inhibitors, SRL has modest or no effects on kidney function, and its effect on direct tubular toxicity has not been demonstrated (1,2). The synergistic effects of SRL and CsA have prompted the investigation of this combination as an immunosuppressive regimen for renal transplant recipients. The use of SRL allows early CsA discontinuation or dose reduction, decreasing nephrotoxicity without increasing the risk of graft loss or acute rejection (6). However, other studies of kidney transplantation have shown that SRL may have long-term adverse subclinical effects or enhance the nephrotoxicity of full doses of CsA (7-10). In addition to drug-induced nephrotoxicity, kidney transplant recipients are at risk of developing ischemia/reperfusion injury, which can influence the clinical outcome and graft survival negatively. Ischemic

Correspondence: L. Yu, Departamento de Nefrologia, LIM 12, Faculdade de Medicina, USP, Av. Dr. Arnaldo, 455, 3º andar, Sala 3310, 01246-903 São Paulo, SP, Brasil. Fax: +55-11-3088-2267. E-mail: luisyu@usp.br

Received March 11, 2010. Accepted May 31, 2010. Available online June 18, 2010. Published August 13, 2010.

injury can be aggravated by CsA due to drug-induced arteriolar vasoconstriction (11). Experimental and clinical studies have provided conflicting results regarding the role of SRL in renal ischemia/reperfusion injury (11-18). Some experimental studies (6,15,16) and retrospective data have suggested that SRL impairs recovery from tubular injury in cases of severe ischemia/reperfusion injury (17). However, two randomized multicenter studies and one large single-center study have not confirmed this effect (12-14). Therefore, whether SRL exacerbates or protects the transplanted kidney from ischemic injury remains to be determined (18).

The molecular mechanisms involved in the potentially nephrotoxic response of tubular cells to immunosuppressive drugs is poorly understood. Transcriptional profiles of human proximal tubular cells exposed to CsA, SRL or their combination have been established using oligonucleotide microarrays (3). Hierarchical clustering of genes implicated in the fibrotic processes showed a clear distinction between expression profiles with CsA and CsA + SRL treatments, especially after biological processes located at the cell membrane level such as ion transport or signal transduction. On the other hand, SRL modifies biological processes within the nucleus, more related to transcriptional activity. Genome-wide expression analysis suggested that CsA may induce an endoplasmic stress in tubular cells *in vitro* (3). More recently, Pallet et al. (3) demonstrated that CsA exposure *in vivo* is associated with up-regulation of the endoplasmic reticulum stress marker in kidney transplant biopsies. The investigators concluded that the toxicogenomic study highlights the molecular interaction network that may contribute to the tubular response to CsA and SRL. These results may also offer a new working hypothesis for future research in the field of CsA nephrotoxicity (3).

The purpose of the present study was to evaluate the effects of SRL alone or in combination with CsA on oxygenated and hypoxic/reoxygenated rat proximal tubules *in vitro*, as well as on renal ischemia/reperfusion injury *in vivo*.

Material and Methods

Animals

Male Wistar rats (3 weeks of age and weighing 150-250 g) obtained from the animal facilities of the University of São Paulo School of Medicine, São Paulo, SP, Brazil, were used in all experimental procedures. The study was approved by the Bioethics Committee of the University of São Paulo School of Medicine, Brazil.

Isolation of proximal tubules

Proximal tubules were isolated using previously described methods (19,20). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, *ip*) and submitted to laparotomy for kidney removal. Renal proximal tubules were isolated by collagenase digestion (Quimis Ltda., Brazil) and separated by Percoll gradient centrifugation (Hitachi, Japan). Aliquots of 6 mL (containing 1.0-1.5 mg/mL protein, Sigma, USA) were placed in siliconized Erlenmeyer flasks for 5 min at ice-cold temperature, under 95% O_2 and 5% CO_2, followed by 10 min at room temperature.

Hypoxia/reoxygenation

After the equilibration period, the isolated tubules were divided into experimental and control samples. Throughout the experiment, pO_2 was maintained in the 200-300-mmHg range in the control group. In the experimental group, hypoxia (pO_2: 20-40 mmHg) was induced by superfusing the tubules with 95% N_2 and 5% CO_2 for 5 min. After 15 min of hypoxia, the tubules were reoxygenated with 95% O_2 and 5% CO_2 for 5 min. After reoxygenation, pO_2 returned to the 200-300-mmHg range. The flasks were then resealed and the tubules maintained under reoxygenation for 45 min. Samples were obtained at baseline, after hypoxia (15 min) and after reoxygenation (60 min).

Determination of lactate dehydrogenase

Cell injury was assessed by the release of lactate dehydrogenase (LDH). In order to determine LDH levels, 1 mL of tubule suspension was centrifuged for 60 s in a refrigerated centrifuge at 3500 *g*. The pellet was lysed with 1.5% Triton X-100. LDH activity was measured in the supernatant and pellet by the method of Bergmeyer (21). LDH activity was converted to percent release by dividing the supernatant activity by the total activity (Hitachi U2000).

Evaluation of the effects of sirolimus alone or combined with CsA on isolated proximal tubules

The effect of various SRL concentrations (10, 50, 100, 250, 500, and 1000 ηg/mL) on isolated proximal tubules was evaluated. Subsequently, the effect of 50 and 250 ηg/mL SRL combined with a concentration of CsA previously determined to be toxic (100 µg/mL) was also evaluated (5). The following groups were studied: control, vehicle (20% ethanol), SRL (10, 50, 100, 250, 500, and 1000 ηg/mL), CsA (100 µg/mL), and SRL + CsA (50 and 250 ηg/mL SRL combined with 100 µg/mL CsA).

Renal ischemia/reperfusion model

Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, *ip*). An abdominal incision was made, and renal vessels were identified on both sides. The left renal pedicle was isolated and ligated, and nephrectomy was performed. The right renal artery was occluded with a microvascular clamp for 30 min; the clamp was then removed in order to reestablish blood flow to the right kidney. The surgical incision was sutured, and rats were allowed to recover, with free access to food and water.

Evaluation of renal function

Renal function was evaluated by the following param-

eters: glomerular filtration rate (GFR) measured by inulin clearance, serum sodium, urinary sodium, fractional excretion of sodium (FENa, expressed as %), urine osmolality, mean arterial pressure, and renal blood flow (RBF). Rats were allocated to the following groups: sham = manipulation of the right renal pedicle plus left nephrectomy; ischemia = right renal artery clamping for 30 min plus left nephrectomy; ischemia plus SRL (I + SRL) = right renal artery clamping for 30 min plus left nephrectomy and administration of SRL (3 mg/kg, *po*); ischemia plus CsA (I + CsA) = right renal artery clamping for 30 min plus left nephrectomy and administration of CsA (3 mg/kg, *sc*); ischemia plus SRL plus CsA (I + SRL + CsA) = right renal artery clamping for 30 min plus left nephrectomy and administration of a combination of SRL (3 mg/kg, *po*) and CsA (3 mg/kg, *sc*).

All drugs were administered 2 days before the surgical procedure and maintained for 24 h (N = 6 for each group) or for 7 days until renal function was evaluated or the kidneys were removed (sham, N = 6; ischemia, N = 6; I + SRL, N = 7; I + CsA, N = 6; I + SRL + CsA, N = 9). All procedures were performed in accordance with our institutional guidelines.

For evaluation of renal function, rats were anesthetized with sodium pentobarbital (50 mg/kg), the trachea was cannulated with a polyethylene catheter (PE-240), and animals were maintained under spontaneous breathing. The jugular veins were cannulated with a PE-60 catheter for infusion of inulin and fluids. The right femoral artery was cannulated with a PE-50 catheter to monitor arterial pressure and for blood sampling. For urine samples, the urinary bladder was cannulated with a PE-240 catheter via a suprapubic incision. RBF was measured by a median incision, the left renal pedicle was carefully dissected, the renal artery was carefully isolated to avoid injury to renal nerves, an electromagnetic flow probe (Transonic Systems, USA) was placed around the exposed renal artery, and RBF was measured using an electromagnetic flowmeter (T 106 XM; Transonic Systems). After the surgical procedure was completed, a loading dose of inulin (100 mg/kg diluted in 1 mL 0.9% saline) was administered through the jugular vein, followed by an infusion of inulin (10 mg/kg in 0.9% saline) at 0.04 mL/min throughout the experimental period. Three urine samples were collected at 30-min intervals. Blood samples were collected at the beginning and at the end of the experiment. Inulin clearance values represent the mean of three periods. Blood and urine inulin were determined by the anthrone method (Merck KGaA, Germany), sodium concentration was measured using a flame photometer, urinary osmolality was measured using a freezing point (model 143; Instrumentation Laboratory, USA), and a fluorescence polarization immunoassay technique (PDX; Abbott, USA) was used to measure blood CsA.

Renal histology

All biopsy samples were fixed in Bouin's medium for 30 min and then immersed in 10% buffered formalin. Samples were processed for histology in an automatic device. Processing consisted of three immersions (1 h each) in absolute ethyl alcohol and three immersions (1 h each) in xylene at room temperature, followed by immersion in two vessels (1 h in each) containing liquid paraffin at approximately 60°C.

After embedding, the samples were blocked in paraffin and cut into 4-µm sections with a microtome. Sections were mounted on slides, deparaffinized in xylene and stained with hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, and Jones' silver stain.

Histomorphometric analysis was performed to evaluate and quantify tubular necrosis in rat renal cortex using hematoxylin and eosin staining. Slides were examined under a light microscope (25X magnification). Examination was performed by a single pathologist, who was blind to the experimental assignments. The following histological lesions were considered to be indicative of tubular necrosis: loss of microvilli, loss of cytoplasmic and nuclear integrity, loss of individual tubular cells, hyaline casts filling the lumen of the proximal tubule, replacing lost tubular cells.

Histomorphometric analysis was performed in a blind manner by a single rater. The presence of acute tubular necrosis (ATN) was estimated in 4-µm sections stained with hematoxylin and eosin. Each histological parameter was evaluated by examining all microscopic fields (15-30 fields in each section) at a final magnification of 250X. A score was attributed to each parameter, as follows: 0 = no lesion observed in the section; 1 = ≤25% of the section affected; 2 = 26-50% of the section affected; 3 = >50% of the section affected.

Statistical analysis

Data are reported as means ± SD. Statistical analysis was performed by analysis of variance and by the Student-Newman-Keuls post-test, with the level of significance set at $P < 0.05$.

Results

In vitro studies

No direct tubular toxicity was observed in isolated proximal tubules even at high SRL concentrations, up to 90 min of incubation (Table 1). Similarly, SRL at concentrations of 250 ηg/mL (45.4 ± 5.9) and 500 ηg/mL (39.6 ± 3.5) did not enhance injury to the proximal tubules submitted to 15 min of hypoxia alone (40.2 ± 9.1), and all groups submitted to hypoxia differed from control (18.6 ± 3.5). Followed by 45 min of reoxygenation, SRL at concentrations of 250 ηg/mL (54.9 ± 5.4) and 500 ηg/mL (48.8 ± 9.6) caused no significant injury to the proximal tubules submitted to hypoxia alone (53.0 ± 9.5), and all groups submitted to hypoxia differed from control (23.4 ± 4.4; Figure 1).

At 60 min, the tubules treated with CsA 100 µg/mL (30.6 ± 7.3), SRL 50 + CsA 100 µg/mL (36.8 ± 10.2) and SRL 250 + CsA 100 µg/mL (33.1 ± 3.5) presented greater LDH

release elevation than tubules treated with SRL alone, 50 ηg/mL (21.0 ± 3.5) and 250 ηg/mL (21.2 ± 6.9), and control (21.5 ± 5.3) (Figure 2). The same effect was observed at 90 min: CsA 100 µg/mL (49.8 ± 11.3), SRL 50 + CsA 100 µg/mL (49.2 ± 8.5) and SRL 250 + CsA 100 µg/mL (52.0 ± 7.6) presented greater LDH release than tubules treated with SRL alone, 50 ηg/mL (31.1 ± 5.6) and 250 ηg/mL (32.4 ± 7.1) and control (29.1 ± 5.9; Figure 2).

In vivo studies

At 24 h, body weight did not differ significantly among the experimental groups. Hematocrit was higher in the I + CsA group compared with the sham and ischemia groups. Mean arterial pressure was higher in the I + SRL + CsA group than in the sham group (Table 2). Despite the reduced RBF and increased renal vascular resistance (RVR) in all groups submitted to ischemia, GFR was not affected in any group. In addition, increased FENa was observed in I + CsA and I + SRL + CsA groups, and reduced urine osmolality was observed in the I + CsA group. Urinary volume was greater in the I + SRL + CsA group than in the sham group ($P < 0.05$; Table 3).

On day 7, body weight was lower in groups I + CsA and I + SRL + CsA than in the ischemia group. Hematocrit was normal and similar among groups. Mean arterial pressure was lower in the I + CsA group than in the sham group (Table 2). A significant reduction in GFR was observed only in the I + CsA group ($P < 0.05$). The decrease in GFR observed in the I + CsA group was not observed in the I + SRL + CsA group. However, SRL provided no protection

Table 1. Effect of sirolimus on isolated proximal tubules as assessed by lactate dehydrogenase release.

Time	Control (N = 13)	Vehicle (N = 8)	Sirolimus (ηg/mL)					
			10 (N = 6)	50 (N = 9)	100 (N = 6)	250 (N = 12)	500 (N = 5)	1000 (N = 6)
Baseline	8.6 ± 2.0	9.0 ± 1.9	8.6 ± 1.1	8.5 ± 2.3	10.1 ± 1.8	9.4 ± 1.7	8.9 ± 1.7	9.5 ± 1.3
60 min	18.6 ± 3.5	19.2 ± 4.5	19.5 ± 2.5	20.4 ± 4.2	21.4 ± 9.8	20.3 ± 6.0	19.9 ± 2.8	21.8 ± 5.1
90 min	23.4 ± 4.4	26.5 ± 8.4	25.3 ± 3.8	28.2 ± 6.0	25.6 ± 4.9	30.1 ± 6.4	26.1 ± 3.0	27.6 ± 5.2

Data are reported as means ± SD lactate dehydrogenase release (%). There were no statistical differences among groups or times (ANOVA followed by the Student-Newman-Keuls post-test).

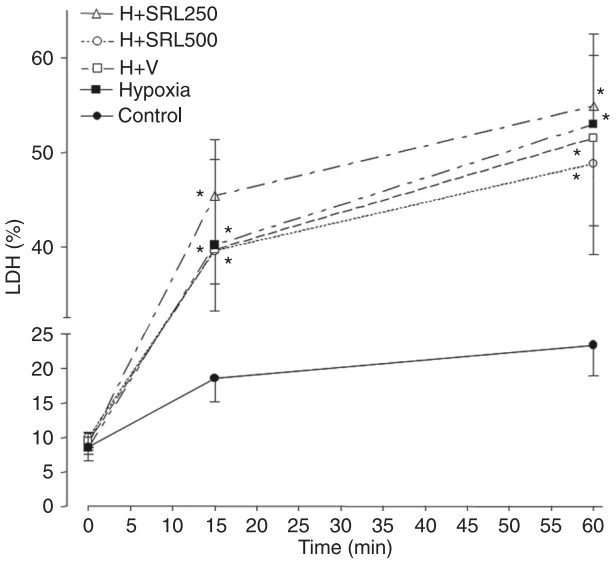

Figure 1. Effect of sirolimus on isolated proximal tubules submitted to 15 min of hypoxia (H) alone or followed by 45 min of reoxygenation, as assessed by release of lactate dehydrogenase (LDH). V = vehicle. Data are reported as mean ± SD. *$P < 0.05$ *vs* control at both 15 and 60 min (ANOVA followed by the Student-Newman-Keuls post-test).

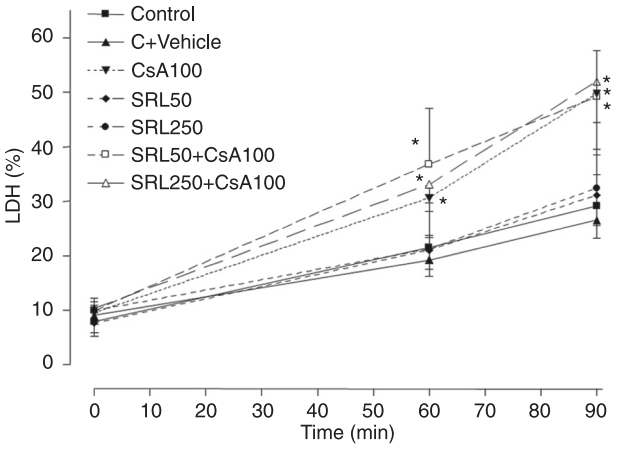

Figure 2. Effect of sirolimus combined with a toxic concentration of cyclosporin A (100 µg/mL) on isolated proximal tubules as assessed by lactate dehydrogenase release (LDH). SRL = sirolimus (ηg/mL); CsA = cyclosporin A (µg/mL). Data are reported as means ± SD. *$P < 0.05$ *vs* control at both 60 and 90 min (ANOVA followed by Student-Newman-Keuls post-test).

against CsA-induced vasoconstriction, since both I + CsA and I + SRL + CsA groups showed a significant reduction in RBF and an increase in RVR. The I + SRL group and the sham group presented similar RBF and RVR. All groups submitted to ischemia, especially the I + CsA group, presented higher FENa. Urine osmolality was higher for sham, I + SRL and I + SRL + CsA groups, and reduced for ischemia and I + CsA groups. Urinary volume was similar for all groups (Table 3).

Histology

At 24 h, foci of ATN (score 2) were found in all groups.

Table 2. Body weight, hematocrit and mean arterial pressure (MAP) in ischemic rats treated with sirolimus (I + SRL), cyclosporine A (I + CsA) and both (I + SRL + CsA).

	Sham	Ischemia	I + SRL	I + CsA	I + SRL + CsA
Body weight (g)					
24 h	209 ± 15	209 ± 8	196 ± 28	222 ± 6	202 ± 10
7 days	230 ± 14	238 ± 13	209 ± 26	199 ± 28^{+}	175 ± 17^{+}
Hematocrit (%)					
24 h	43.0 ± 4.1	41.6 ± 5.8	48.3 ± 6.3	55.1 ± 10.7^{*+}	49.1 ± 7.6
7 days	48.3 ± 3.6	51.0 ± 2.0	47.2 ± 18	44.1 ± 7.0	48.3 ± 8.4
MAP (mmHg)					
24 h	114 ± 7	118 ± 12	117 ± 9	111 ± 13	130 ± 10*
7 days	125 ± 7	117 ± 12	120 ± 15	107 ± 19*	128 ± 12

Data are reported as means ± SD. All drugs were administered 2 days before the surgical procedure and maintained for 24 h or for 7 days. Doses: SRL (3 mg·kg^{-1}·day^{-1}, *po*) and CsA (3 mg·kg^{-1}·day^{-1}, *sc*). *P < 0.05 compared to the sham group; $^{+}$P < 0.05 compared to the ischemia group (ANOVA followed by the Student-Newman-Keuls post-test).

Table 3. Renal function and hemodynamic parameters in ischemic rats treated with sirolimus (I + SRL), cyclosporine A (I + CsA) and both (I + SRL + CsA).

	Sham	Ischemia	I + SRL	I + CsA	I + SRL + CsA
GFR (mL/min)					
24 h	0.8 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.8 ± 0.3	0.8 ± 0.2
7 days	0.8 ± 0.1	0.7 ± 0.09	0.7 ± 0.2	0.5 ± 0.1*	0.8 ± 0.1
RBF (mL/min)					
24 h	7.1 ± 0.6*	4.5 ± 0.8	4.5 ± 0.9	5.5 ± 1.0	5.1 ± 1.0
7 days	7.9 ± 0.2	7.5 ± 0.2	6.8 ± 0.7	4.4 ± 2.2^{*+}	3.9 ± 0.5^{*+}
RVR (mmHg·mL^{-1}·min^{-1})					
24 h	16.3 ± 2.3	26.7 ± 6.8**	26.7 ± 5.8**	20.5 ± 4.8	26.2 ± 5.2**
7 days	16.1 ± 1.1	15.5 ± 1.9	18.5 ± 2.6	33.4 ± 27.2^{*+}	32.9 ± 6.1^{*+}
FeNa (%)					
24 h	0.8 ± 0.7	2.4 ± 1.7	2.1 ± 1.3	3.4 ± 1.4**	3.2 ± 1.9**
7 days	0.4 ± 0.4	1.3 ± 0.9	2.2 ± 1.5*	5.2 ± 3.8^{*+}	1.5 ± 0.4
Uosm (mmOsm/kg)					
24 h	929 ± 279	606 ± 130**	838 ± 144	586 ± 313**	771 ± 163
7 days	1333 ± 424	895 ± 286*	913 ± 186*	1115 ± 325	1015 ± 274
UV (mL/min)					
24 h	0.02 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.06 ± 0.02	0.05 ± 0.02**
7 days	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.05	0.03 ± 0.02	0.03 ± 0.03

Data are reported as means ± SD. GFR = glomerular filtration rate; RBF = renal blood flow; RVR = renal vascular resistance; FENa = fractional excretion of sodium; Uosm = urine osmolality; UV = urinary volume. All drugs were administered 2 days before the surgical procedure and maintained for 24 h or for 7 days. Doses: SRL (3 mg·kg^{-1}·day^{-1}, *po*) and CsA (3 mg·kg^{-1}·day^{-1}, *sc*). *P < 0.05 compared to the other groups; **P < 0.05 compared to the sham group; $^{+}$P < 0.05 compared to the ischemia group (ANOVA followed by the Student-Newman-Keuls post-test).

This alteration was less pronounced in the I + CsA group and more pronounced in the ischemia group. On day 7, foci of ATN remained in groups I + CsA and I + SRL + CsA and no ATN was observed in the other groups (Table 4 and Figure 3).

Blood concentration of cyclosporin A

There was no significant difference in blood CsA concentration between I + CsA and I + SRL + CsA groups 24 h after drug administration (298 ± 29 *vs* 304 ± 36 mg/dL; P > 0.05).

Discussion

SRL is an immunosuppressant with a specific mechanism of action and negligible nephrotoxicity (22,23). In addition, SRL can synergize with other immunosuppressants such as calcineurin inhibitors without additional side effects. Because of these characteristics, there has been considerable interest in the use of SRL in organ transplantation and autoimmune diseases (23). In contrast to the direct tubular toxicity demonstrated for CsA (5), we found that SRL had no effect on isolated proximal tubules, either in normoxia or in hypoxia/reoxygenation injury. These results reinforce the idea that sirolimus has no direct nephrotoxicity. In addition, the combination of SRL with CsA did not enhance the direct tubular toxicity of CsA. This can be attributed to the difference in the intracellular activity of these two drugs, which distinguishes SRL from calcineurin inhibitors (1,5,23,24).

No significant change in GFR was observed at 24 h, probably due to the short-term ischemia associated with

Table 4. Histomorphometric analysis in ischemic rats treated with sirolimus (I + SRL), cyclosporine A (I + CsA) and both (I + SRL + CsA).

	Sham	Ischemia	I + SRL	I + CsA	I + SRL + CsA
Score 1					
24 h	50.0	33.4	60.0	66.6	50.0
7 days	100.0	50.0	85.7	57.1	28.6
Score 2					
24 h	50.0	16.7	20.0	22.2	16.7
7 days	0	50.0	14.3	28.6	57.1
Score 3					
24 h	0	50.0	20.0	11.2	33.3
7 days	0	0	0	14.3	14.3

Data are reported as percent. Score 1: ≤25% of the section was affected; Score 2: 26-50% of the section was affected; Score 3: >50% of the section was affected. All drugs were administered 2 days before the surgical procedure and maintained for 24 h or for 7 days. Doses: SRL (3 $mg \cdot kg^{-1} \cdot day^{-1}$, *po*) and CsA (3 $mg \cdot kg^{-1} \cdot day^{-1}$, *sc*).

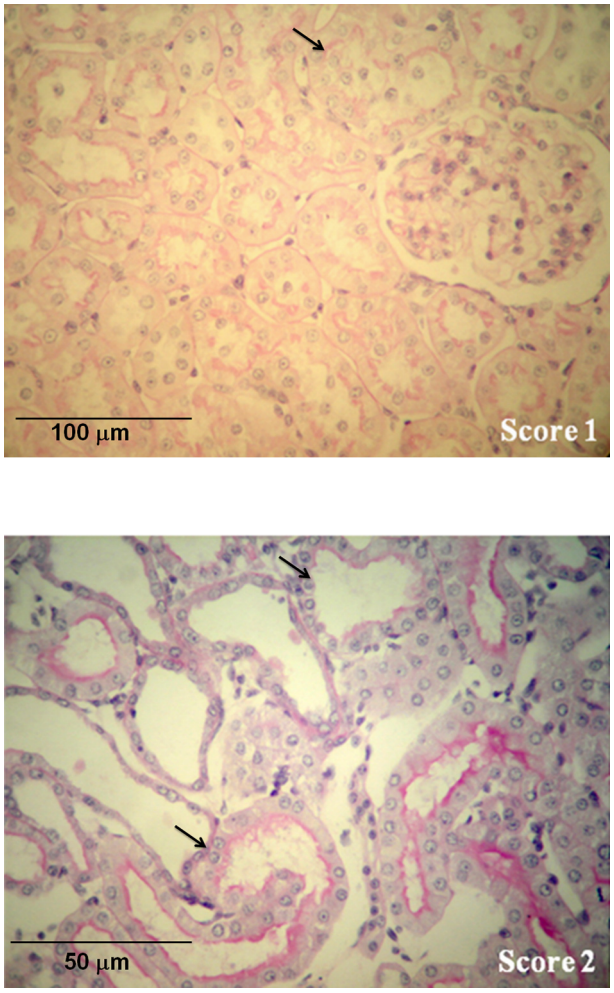

Figure 3. Illustration showing the characteristics of the three scores used to evaluate acute tubular necrosis (arrows): score 1 (lesions in ≤25% of the section), score 2 (lesions in 26-50% of the section), score 3 (lesions in >50% of the section).

unilateral nephrectomy, which reduces acute renal insufficiency. The combination of SRL and CsA caused mild tubular injury demonstrated by the increased FENa at 24 h and the decreased urinary osmolality of the group treated only with CsA (Table 3). On day 7, only the I + CsA group presented tubular dysfunction and reduced GFR (Table 3), which was attenuated by the combination with SRL. This finding corroborates those of clinical studies that have shown that a combination of SRL with low doses of CsA has a beneficial effect on graft survival (25,26). However, experimental studies have yielded contradictory results (26,27). Burdmann et al. (27) demonstrated that there was an increase in enzymuria 14 days after CsA or FK506 administration, which did not occur with SRL. Whiting et al. (28) showed that SRL and CsA together caused a significant increase in enzymuria and a reduction of GFR, both effects attributable to additive renal toxicity (29,30). Lieberthal et al. (15) demonstrated that recovery of renal function was delayed in rats treated with SRL on days 3 and 4 after 40-min ischemia. Using a model of chronic nephrotoxicity, Shihab et al. (30) found that SRL alone did not affect renal function, but SRL in combination with low doses of CsA presented a nephrotoxic effect, which was attributed to a greater TGF-β1 expression. In the present study, we observed tubular changes but no GFR alterations, suggesting that SRL might have an initial toxic effect that was undetectable after 7 days of treatment, when its protective effect against CsA toxicity was observed.

At 24 h, hemodynamic alterations were observed in all groups submitted to ischemia (Table 3). This finding was probably due to vasoconstriction, a common event occurring immediately after ischemia, caused in part by the activation of the tubuloglomerular feedback. Vasoconstriction can also be caused by increased basal vascular tone, by enhanced reactivity to vasoconstrictors or by the reduced vasodilator response observed in post-ischemic kidneys (25). Hemodynamic changes such as increased RVR and reduced RBF had reverted in all groups by day 7, except in those receiving CsA. However, although RVR and RBF values did not revert in the I + SRL + CsA group, the GFR of this group was comparable to that of non-CsA groups (Table 3). Therefore, the GFR reduction observed in the I + CsA group might have been due to CsA-induced vasoconstriction. These acute effects of CsA have been described (28,31). Sabbatini et al. (32) demonstrated that immunosuppressant doses of CsA were toxic, whereas SRL, even at high doses, had a negligible effect on renal and glomerular dynamics. L'Azou et al. (31) demonstrated that CsA nephrotoxicity was mediated by mesangial cell contraction and a consequent decrease in the capillary filtration coefficient. We believe that the preserved GFR in the I + SRL + CsA group was probably due to the SRL-induced increase in capillary filtration coefficient, since glomerular filtration was restored despite RBF reduction. Our findings agree with other studies, which have reported that the combination of CsA and SRL may have a beneficial, synergistic immunosuppressive effect (without additional nephrotoxic effects), which might result in improved post-transplant renal function (9,10,26,33). The protection provided by SRL against CsA nephrotoxicity in ischemia/reperfusion injury cannot be attributed to reduced blood concentrations of CsA since blood levels of CsA were comparable between groups I + CsA and I + SRL + CsA. Similar findings have been reported by Whiting et al. (28).

Foci of ATN were found in all groups after 24 h, although less pronounced in the CsA group. Histological analysis performed on day 7 showed partial recovery from ATN, although less pronounced in I + CsA and I + SRL + CsA groups. No correlation was observed between histological and functional findings in the present study. However, score 1 and score 2 lesions were more prevalent in the groups receiving CsA (Table 4). It is also noteworthy that the tubular vacuolization, nephrocalcinosis, tubular atrophy, and interstitial fibrosis observed in another study, in which CsA was administered for 15 days or more (2), were not observed in the present study.

In conclusion, SRL had no direct toxic effects and did not enhance hypoxia/reoxygenation injury or CsA toxicity in isolated renal proximal tubules. SRL also did not affect GFR after ischemia and prevented CsA-induced toxicity in the ischemic kidney.

References

1. Sehgal SN. Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. *Clin Biochem* 1998; 31: 335-340.
2. Andoh TF, Lindsley J, Franceschini N, Bennett WM. Synergistic effects of cyclosporine and rapamycin in a chronic nephrotoxicity model. *Transplantation* 1996; 62: 311-316.
3. Pallet N, Rabant M, Xu-Dubois YC, Lecorre D, Mucchielli MH, Imbeaud S, et al. Response of human renal tubular cells to cyclosporine and sirolimus: a toxicogenomic study. *Toxicol Appl Pharmacol* 2008; 229: 184-196.
4. Nankivell BJ, Chapman JR. Chronic allograft nephropathy: current concepts and future directions. *Transplantation* 2006; 81: 643-654.
5. Carvalho da Costa M, de Castro I, Neto AL, Ferreira AT, Burdmann EA, Yu L. Cyclosporin A tubular effects contribute to nephrotoxicity: role for Ca^{2+} and Mg^{2+} ions. *Nephrol Dial Transplant* 2003; 18: 2262-2268.
6. Ysebaert DK, De Greef KE, Nouwen EJ, Verpooten GA, Eyskens EJ, De Broe ME. Influence of cyclosporin A on the damage and regeneration of the kidney after severe ischemia/reperfusion injury. *Transplant Proc* 1997; 29: 2348-2351.

7. MacDonald AS. A worldwide, phase III, randomized, controlled, safety and efficacy study of a sirolimus/cyclosporine regimen for prevention of acute rejection in recipients of primary mismatched renal allografts. *Transplantation* 2001; 71: 271-280.
8. Kahan BD. Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomised multicentre study. The Rapamune US Study Group. *Lancet* 2000; 356: 194-202.
9. Hsu HJ, Tian YC, Chen YC, Lai BC, Fang JT, Yang CW, et al. The combination of sirolimus and cyclosporine does not delay initial renal graft function recovery. *Ren Fail* 2008; 30: 303-306.
10. Lebranchu Y, Thierry A, Toupance O, Westeel PF, Etienne I, Thervet E, et al. Efficacy on renal function of early conversion from cyclosporine to sirolimus 3 months after renal transplantation: concept study. *Am J Transplant* 2009; 9: 1115-1123.
11. Inman SR, Davis NA, Olson KM, Lukaszek VA, McKinley MR, Seminerio JL. Rapamycin preserves renal function compared with cyclosporine A after ischemia/reperfusion injury. *Urology* 2003; 62: 750-754.
12. Mendez R, Gonwa T, Yang HC, Weinstein S, Jensik S, Steinberg S. A prospective, randomized trial of tacrolimus in combination with sirolimus or mycophenolate mofetil in kidney transplantation: results at 1 year. *Transplantation* 2005; 80: 303-309.
13. Morales JM, Wramner L, Kreis H, Durand D, Campistol JM, Andres A, et al. Sirolimus does not exhibit nephrotoxicity compared to cyclosporine in renal transplant recipients. *Am J Transplant* 2002; 2: 436-442.
14. Knight RJ, Kerman RH, Schoenberg L, Podder H, Van Buren CT, Katz S, et al. The selective use of basiliximab versus thymoglobulin in combination with sirolimus for cadaveric renal transplant recipients at low risk versus high risk for delayed graft function. *Transplantation* 2004; 78: 904-910.
15. Lieberthal W, Fuhro R, Andry CC, Rennke H, Abernathy VE, Koh JS, 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.
16. Ninova D, Covarrubias M, Rea DJ, Park WD, Grande JP, Stegall MD. Acute nephrotoxicity of tacrolimus and sirolimus in renal isografts: differential intragraft expression of transforming growth factor-beta1 and alpha-smooth muscle actin. *Transplantation* 2004; 78: 338-344.
17. McTaggart RA, Gottlieb D, Brooks J, Bacchetti P, Roberts JP, Tomlanovich S, et al. Sirolimus prolongs recovery from delayed graft function after cadaveric renal transplantation. *Am J Transplant* 2003; 3: 416-423.
18. Knight RJ, Kahan BD. The place of sirolimus in kidney transplantation: can we reduce calcineurin inhibitor renal toxicity? *Kidney Int* 2006; 70: 994-999.
19. Yu L, Gengaro PE, Niederberger M, Burke TJ, Schrier RW. Nitric oxide: a mediator in rat tubular hypoxia/reoxygenation injury. *Proc Natl Acad Sci U S A* 1994; 91: 1691-1695.
20. Gesek FA, Wolff DW, Strandhoy JW. Improved separation method for rat proximal and distal renal tubules. *Am J Physiol* 1987; 253: F358-F365.
21. Bergmeyer HU. *Methods of enzymatic analysis*. 2nd edn. New York: Academic Press; 1974.
22. Warner LM, Adams LM, Chang JY, Sehgal SN. A modification of the *in vivo* mixed lymphocyte reaction and rapamycin's effect in this model. *Clin Immunol Immunopathol* 1992; 64: 242-247.
23. Groth CG, Backman L, Morales JM, Calne R, Kreis H, Lang P, et al. Sirolimus (rapamycin)-based therapy in human renal transplantation: similar efficacy and different toxicity compared with cyclosporine. Sirolimus European Renal Transplant Study Group. *Transplantation* 1999; 67: 1036-1042.
24. Schuler W, Sedrani R, Cottens S, Haberlin B, Schulz M, Schuurman HJ, et al. SDZ RAD, a new rapamycin derivative: pharmacological properties *in vitro* and *in vivo*. *Transplantation* 1997; 64: 36-42.
25. Flechner SM, Goldfarb D, Modlin C, Feng J, Krishnamurthi V, Mastroianni B, et al. Kidney transplantation without calcineurin inhibitor drugs: a prospective, randomized trial of sirolimus versus cyclosporine. *Transplantation* 2002; 74: 1070-1076.
26. Johnson RW, Kreis H, Oberbauer R, Brattstrom C, Claesson K, Eris J. Sirolimus allows early cyclosporine withdrawal in renal transplantation resulting in improved renal function and lower blood pressure. *Transplantation* 2001; 72: 777-786.
27. Burdmann EA, Andoh TF, Lindsley J, Russell J, Bennett WM, Porter G. Urinary enzymes as biomarkers of renal injury in experimental nephrotoxicity of immunosuppressive drugs. *Ren Fail* 1994; 16: 161-168.
28. Whiting PH, Woo J, Adam BJ, Hasan NU, Davidson RJ, Thomson AW. Toxicity of rapamycin - a comparative and combination study with cyclosporine at immunotherapeutic dosage in the rat. *Transplantation* 1991; 52: 203-208.
29. Andoh TF, Burdmann EA, Fransechini N, Houghton DC, Bennett WM. Comparison of acute rapamycin nephrotoxicity with cyclosporine and FK506. *Kidney Int* 1996; 50: 1110-1117.
30. Shihab FS, Bennett WM, Yi H, Choi SO, Andoh TF. Sirolimus increases transforming growth factor-beta1 expression and potentiates chronic cyclosporine nephrotoxicity. *Kidney Int* 2004; 65: 1262-1271.
31. L'Azou B, Medina J, Frieauff W, Cordier A, Cambar J, Wolf A. *In vitro* models to study mechanisms involved in cyclosporine A-mediated glomerular contraction. *Arch Toxicol* 1999; 73: 337-345.
32. Sabbatini M, Sansone G, Uccello F, De Nicola L, Nappi F, Andreucci VE. Acute effects of rapamycin on glomerular dynamics: a micropuncture study in the rat. *Transplantation* 2000; 69: 1946-1990.
33. Wyzgal J, Paczek L, Senatorski G, Zygier J, Rowinski W, Szmidt J, et al. Sirolimus rescue treatment in calcineurin-inhibitor nephrotoxicity after kidney transplantation. *Transplant Proc* 2002; 34: 3185-3187.