View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Kidney International, Vol. 48 (1995), pp. 56–64

FK 506: Effects on glomerular hemodynamics and on mesangial cells in culture

SEMIRAMIS J. HADAD, EDISON R.M. SOUZA, ALICE T. FERREIRA, MARIA E.M. OSHIRO, MIRIAN A. BOIM, CLARA V. RAZVICKAS, LUIZ A.R. MOURA, and NESTOR SCHOR

Nephrology Division, and Biophysics and Pathology Departments, Escola Paulista de Medicina, São Paulo, Brasil

FK 506: Effects on glomerular hemodynamics and on mesangial cells in culture. FK 506 is a new immunosuppressive drug that, like cyclosporine A (CsA), presents nephrotoxicity. Glomerular hemodynamic studies showed that acute FK 506 infusion ($N = 9$, 3 mg/kg body wt, i.v. in bolus) caused a 57% reduction in glomerular filtration rate (GFR) (0.74 ± 0.03 to 0.32 ± 0.02 ml/min, $P < 0.05$) and a 40% reduction in single nephron glomerular filtration rate (SNGFR; 43.0 ± 5.2 to 26.0 ± 2.5 nl/min, $P < 0.05$) due to a 25% reduction in glomerular plasma flow rate (Q_A) (133.4 ± 19.8 to 99.8 ± 12.0 nl/min) and a 22% reduction in glomerular ultrafiltration coefficient (K_f; 0.1009 ± 0.0203 to 0.0790 ± 0.0130 nl/sec · mm Hg). After 10 days of FK treatment ($N = 8$, 0.6 mg/kg body wt, i.p.), we observed a reduction of 23% in GFR (0.97 ± 0.02 to 0.75 ± 0.04 ml/min, $P < 0.05$) and of 23% in SNGFR (37.9 ± 3.0 to 29.1 ± 1.9 nl/min, $P < 0.05$) due to a 42% reduction in K_f (0.1486 ± 0.0101 to 0.0870 ± 0.0110 nl/sec · mm Hg, $P < 0.05$) and a 38% reduction in Q_A (117.6 ± 10.2 to 73.5 ± 6.1 nl/min, $P < 0.05$). The latter was consequent to the increment of 72% in total arteriolar resistance (R_T) (3.1 ± 0.2 to 5.2 ± 0.5 ± 0.5 10^{10} · dyn · sec · cm^{-5}, $P < 0.05$).Thus, the pattern of FK 506 effect on glomerular hemodynamics was similar in both acute and chronic treatments. Additionally, in order to evaluate the effect of FK 506 on mesangial cells (MC), we performed studies measuring intracellular calcium concentration ($[Ca^{2+}]_i$) with Fura-2/AM as well as MC contraction by morphometric analysis. It was observed that FK 506 increases the $[Ca^{2+}]_i$ (R 340/380: 2.5 ± 0.3 to 3.8 ± 0.4, $P < 0.05$) due to mobilization of the extracellular calcium pool, via opening calcium type L voltage dependent channels, since verapamil blunted the increases of $[Ca^{2+}]_i$ caused by FK 506 (R 340/380: 3.5 ± 0.9 to 2.8 ± 0.8). The $[Ca^{2+}]_i$ was not changed after FK 506 incubation of MC with verapamil and thapsigargin, and thus no calcium release-activated channel (CRAC) was affected. The increase of $[Ca^{2+}]_i$ induced by FK 506 probably caused contraction of MC evaluated by reduction of the cross sectional area from 3772 ± 106 to 1912 ± 61 μm^2 ($P < 0.05$). Thus, FK 506 caused a reduction in SNGFR by reducing Q_A and K_f after both acute and chronic administration, and the mesangial cells potentially participate in this nephrotoxicity via reduction in K_f, mainly during chronic treatment.

FK 506 (FK) is a new immunosuppressive drug that, like cyclosporine A (CsA), presents side effects including nephrotoxicity. Functionally, both drugs induce acute renal failure due to reduction in renal plasma flow (RPF) and in glomerular filtration rate (GFR) [1–5]. The hemodynamic events related to FK may include the vasoconstriction of glomerular arterioles, the liberation of vasoactive substances and the contraction of mesangial cells (MC) [1–3, 6, 7]. Additionally, the cellular events triggered by FK and CsA seem to be similar. The nonspecific action of these drugs on the immune system cells results from the interaction with intracellular receptors, generically recognized as immunophilins. Cyclophilin and FK-binding protein (FKBP) are the immunophilins used for CsA and FK, respectively. The common target for both complexes is the calcineurin present in many cells [8, 9]. *In vitro*, calcineurin binds to the FK-FKBP complex in a Ca^{2+}-dependent manner. Thus, FK blocks a Ca^{2+}-dependent signaling step that normally leads to nuclear transport of a cytoplasm subunit of nuclear factor activated T cells (NF-AT) which is one of many transcription factors required for antigen-induced gene expression [9]. It is known that FK binding protein is found in many live cells from different animal species and thus FK may potentially interfere with these cells, explaining the side effects observed.

The contraction of MC induced by the increases of intracellular calcium concentration ($[Ca^{2+}]_i$) causes a decrease in glomerular surface area and in glomerular ultrafiltration coefficient (K_f); thus it could be a potential determinant of nephrotoxicity in animal studies [10–13].

Although many effects of FK have been clarified, its actions on the renal microcirculation and on MC have not yet been evaluated. Thus, the objective of the present investigation was to study glomerular hemodynamics during acute and chronic treatment with FK and to determine the action of the drug on $[Ca^{2+}]_i$ and on MC surface area.

Methods

Hemodynamic studies

Studies were performed on four groups of adult male Munich-Wistar rats weighing 200 to 300 g. Group 1 ($N = 5$) received an acute infusion of FK vehicle, cremophor and isopropilic alcohol i.v. for five minutes. Group 2 ($N = 9$), received FK, 3 mg/kg body wt, i.v., in bolus (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan). Parameters of whole kidney function and glomerular hemodynamics were measured before and after the acute infusions. The third group (Group 3, $N = 8$) and the fourth group (Group 4, $N = 9$) received chronic i.p. administration of FK vehicle or FK, 0.6 mg/kg body wt, for 10 days, respectively. After 10 days the animals were submitted to experimental studies to evaluate whole kidney function and glomerular hemodynamics.

Received for publication August 19, 1994
and in revised form February 17, 1995
Accepted for publication February 21, 1995

© 1995 by the International Society of Nephrology

Micropuncture studies were performed in all rats following the usual routine methods [11, 12, 14]. Each rat was allowed free access to water and a standard rat pellet diet until the morning of the study. Rats were anesthetized with inactin (BYC Gulden, Konstaz, Germany), 100 mg/kg body wt, i.p. and placed on a heated animal board in order to maintain rectal temperature close to 37°C. Following tracheotomy, a polyethylene catheter (PE-50) was introduced into the left femoral artery and connected to a direct-writing recorder (Gould model 2200, Cleveland, OH, USA) for mean arterial pressure (MAP) measurements and collection of blood samples. Approximately 60 μl of arterial blood was collected for baseline hematocrit (Hct) and protein (C_A) measurements. The left jugular vein was catheterized (PE-50) for infusion of inulin (10%) and *p*-aminohippuric acid (PAH) (2%) at a rate of 1.2 ml/hr, and the catheter was maintained throughout the experiment to determine glomerular filtration rate (GFR) and total renal plasma flow rate (RPF). The right jugular vein was catheterized (PE-50) for infusion of isoncotic rat serum to replace loss of liquids during surgery and to maintain Hct at preoperative levels [15]. The infusion rate was 10 ml/kg/hr for 45 minutes and was then reduced to 1.5 ml/kg/hr for the remainder of the experiment. After laparotomy, a catheter (PE-10) was inserted into the left ureter for urine collection and flow rate (V) determination. After a 45-minute equilibration period, the initial study period was started. Two urine and blood samples were collected for determination of Hct, C_A, inulin and PAH in plasma and urine, and the procedure was repeated during the second study period.

Samples of fluid surface proximal tubules were collected for determination of flow rate and inulin concentration, followed by calculation of single nephron glomerular filtration rate (SNGFR). Hydraulic pressures were measured in surface glomerular capillaries (P_{GC}), proximal tubules (P_T), efferent arterioles (P_{EA}) and peritubular capillaries (P_C) with a continuous recording servonull micropipette transducer system (IPM Inc., San Diego, CA, USA). The results are reported as mean ± SEM. To estimate the pre- and postglomerular colloid osmotic pressures, π_A and π_E, protein concentration in femoral arterial (C_A) and efferent arteriolar (C_E) plasma were measured as described previously [16]. These estimations permitted the calculation of single nephron filtration fraction (SNFF), initial glomerular capillary plasma flow rate (Q_A), afferent (R_A), efferent (R_E) and total arteriolar resistance (R_T) and glomerular ultrafiltration coefficient (K_f), using equations previously described [16, 17]. Data were corrected for the left kidney weight in grams. The total renal vascular resistance (TRVR) was calculated according to the formula TRVR = (MAP − 3)/[RPF/(1 − Hct)] [14].

Analytical procedures. Plasma and urinary inulin concentrations were determined by the macroanthrone method of Fuhr, Kaczmarczyk and Kruttgen [18]. PAH concentration was determined by the method of Smith et al [19]. Inulin concentration in tubular fluid was measured by the microfluorescence method [20]. Protein concentrations in efferent arteriolar and femoral arterial plasma were determined by the fluorimetric method [21]. Whole kidney filtration fraction (FF) was calculated as the GFR/RPF ratio.

At the end of the functional studies the kidneys from the chronic groups were fixed by intravascular perfusion of a solution containing phosphate-buffered 2% glutaraldehyde, pH 7.2 [22]. Paraffin-embedded tissue was cut into 3 to 5 μm sections which were stained with H.E., PAS and Masson trichrome and examined by light microscopy.

Mesangial cell culture

Materials. RPMI 1640, collagenase Ia, fetal calf serum, penicillin G, glutamine, acid HEPES, bovine albumin, trypsin, verapamil, thapsigargin, digitonin, manganese, EGTA and fibronectin antibody, actin, myosin, collagen IV, citokeratin and factor VIII were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fura-2/AM was obtained from Molecular Probe (OR, USA) and FK 506, Tacrolimus®, was a gift from Fugisawa Pharmaceutical Co. Ltd. (Osaka, Japan).

Methods. The glomeruli were isolated from Wistar rats by the method of Greenspon and Krakower [23]. Briefly, rats were anesthetized with sulfuric ether and submitted to bilateral nephrectomy. The separated cortex was sliced and forced through a graded series of stainless steel meshes, and isolated uncapsulated glomeruli were recovered. MC were obtained from collagenase-treated isolated glomeruli in order to remove the epithelial cell component. Washed glomerular remnants were plated at a density of about 300 glomeruli/cm^2 onto RPMI 1640 supplemented with 20% fetal calf serum, 50 U/ml penicillin, 2.6 g acid HEPES and 2 mM glutamine. Culture flasks were kept in a 95% air, 5% CO_2 humidified environment at 37°C. The medium was replaced every 36 hours. After three weeks in primary culture, MC were harvested with trypsin. Subcultures were grown in the same medium. These cells were characterized by the following criteria: (a) morphological appearance of stellate cells; (b) immunofluorescence staining of the extracellular matrix for type IV collagen and fibronectin using monospecific antiserum; (c) negative immunofluorescence staining for human factor VIII antigens and citokeratin; and (d) positive immunofluorescence staining for actin and myosin monospecific antiserum. In parallel experiments, cell viability was monitored with Trypan blue and was observed to be about 95%.

$[Ca^{2+}]_i$ determination. Cultures in 3rd passage were treated with 0.05% trypsin after 24 hours in fetal bovine serum free medium and resuspended in Tyrode solution containing 0.2% bovine albumin. The cell suspension was left to stand for 60 minutes in a CO_2 incubator at 37°C. $[Ca^{2+}]_i$ was measured ratiometrically after loading the cells with the Ca^{2+}-sensitive dye Fura-2 using a SPEX fluorimeter (AR CM System, NJ, USA). Cells were centrifuged and resuspended in albumin-free Tyrode. A 2.5 ml volume with 10^6 cells/cm^3 was transferred to a quartz cuvette and submitted to constant stirring at 37°C in a fluorimeter calibrated with excitation at two different wavelengths (340 and 380 nm) and 505 nm emission. Cell autofluorescence was first measured and considered to be negligible when lower than 10%. Cells were later incubated with Fura-2/AM (2 μM) for three hours before the beginning of the experiment. After analysis of the fluorescence spectrum to confirm appropriate indicator loading, the cells were washed with Tyrode, resuspended in fresh solution and placed in quartz cuvettes. Six groups were studied for FK action on MC. The effect of FK vehicle was first tested on Fura-2/AM fluorescence and on $[Ca^{2+}]_i$. Both were negative and permitted continuation of the experiment.

Group 1 ($N = 8$): We analyzed five doses (D) of FK successively given after stabilization: D_1, 10^{-7} M; D_2, 2×10^{-7} M; D_3, 4×10^{-7} M; D_4, 5×10^{-7} M; and D_5, 10^{-6} M.

Group 2 ($N = 4$): The effect of successive doses of FK (D_1, 10^{-7}

Table 1. Summary of general parameters and whole kidney function in the acute groups before and after infusion of vehicle (VEH) or FK-506 (FK)

Group	BW	KW	MAP	Hct	$\dot{V}$	GFR	RPF	FF	TRVR
	g		*mm Hg*	*%*	*μl/min*	*ml/min*		*%*	*mm Hg·min/ml*
Acute VEH ($N = 5$)									
before	—	—	116	49	4.0	0.80	2.28	35	24
			± 2	± 1	± 0.5	± 0.01	± 0.87	± 1	± 1
after	259	0.84	110	49	3.9	0.76	2.21	36	24
	± 12	± 0.06	± 2	± 1	± 0.5	± 0.02	± 0.06	± 1	± 1
Acute FK ($N = 9$)									
before	—	—	102	48	3.0	0.74	2.05	36	22
			± 2	± 1	± 0.5	± 0.03	± 0.08	± 1	± 1
after	247	0.96	99	48	2.7	0.32[a]	0.84[a]	36	54[a]
	± 5	± 0.02	± 3	± 2	± 0.6	± 0.02	± 0.06	± 3	± 6

Data are X ± SEM.
[a] $P < 0.05$ after vs. before

M; D_2, 2×10^{-7} M; D_3, 4×10^{-7} M; D_4, 5×10^{-7} M; and D_5, 10^{-6} M) on $[Ca^{2+}]_i$ was studied after incubation with verapamil (VP, 5×10^{-5} M) for 10 minutes.

Group 3 ($N = 4$): The effect of successive doses of FK (D_1, 10^{-7} M; D_2, 2×10^{-7} M; D_3, 4×10^{-7} M; D_4, 5×10^{-7} M; and D_5, 10^{-6} M) was evaluated on MC in Ca^{2+}-free buffer. After the last dose of FK, $CaCl_2$ was added to the solution in order to obtain a final concentration of 1.36 mM in the bath and fluorescence was recorded again. After stabilization of the curve a new dose of 1.36 mM calcium was added, increasing the bath final concentration to 2.7 mM.

Group 4 ($N = 3$): The effect of 10^{-6} M FK was evaluated after incubation of MC with thapsigargin (TSG, 2 μM) in a solution with normal calcium values.

Group 5 ($N = 2$): The protocol was the same as for Group 4 (FK 10^{-6} M) except that the MC were tested in calcium-free Tyrode solution with TSG added. After this effect was recorded, two successive doses of 1.36 mM $CaCl_2$ separated by a period of stabilization were added, and a further FK in maximal dose was also added.

Group 6 ($N = 2$): The action of FK (10^{-6} M) was evaluated after incubation with TSG and VP in a calcium-free MC suspension. Again, two successive doses of 1.36 mM calcium were added and a further FK in maximal dose was also added.

At the end of each experiment, the effect of digitonin, Mn and EGTA was evaluated for control of the preparation. The results are presented as the 340/380 ratio and when necessary this ratio was transformed in order to provide the calcium concentration as described by Grynkiewicz et al [24].

Morphometry

Mesangial cells were evaluated morphometrically after 10 minutes incubation with the maximum FK dose (10^{-6} M) and after staining with acridine orange. Cells were then photographed and the measurements calculated on a digital board using the Sigmanscan software (Sigmanscan®, Jandel Scientific, Sausalito, CA, USA). The calibration was done according to photograph magnification. Results are reported as μm^2 and were compared in terms of percent modification of the area in comparison to the control. A > 10% reduction in area in relation to the control was considered to represent cell contraction [25].

Statistical analyses

Data for *in vivo* studies are presented as mean (X) ± SEM. Data for whole kidney function were normalized for left kidney weight in grams. Statistical analyzes for global and hemodynamic studies were performed by Student's paired and unpaired *t*-test. Statistical significance was defined as $P < 0.05$. For *in vitro* studies with mesangial cells, the results were analyzed by the Wilcoxon test for group 1, with $P < 0.05$ considered to be significant. Results obtained for intracellular calcium were expressed as the mean of the 340/380 ratios. Data for groups 2, 3 and 4 were analyzed by Friedman two-way rank analysis of variance [26], with $P < 0.05$ considered to be significant. For groups 5 and 6, no statistical tests were utilized.

The morphometric data were analyzed by the Mann Whitney test for unpaired data obtained before and after incubation with the maximal FK dose. A $P < 0.05$ was considered significant.

Results

Table 1 shows general and whole kidney function parameters in the acute groups, before and after infusions of FK or FK vehicle. Acutely administered FK vehicle (Group 1) did not change MAP, V, GFR, RPF, FF or TRVR. Conversely, the acute infusion of FK (Group 2) induced an important decline in GFR (57%) and RPF (59%) (Fig. 1). The filtration fraction (FF) remained stable and TRVR increased 2.5 times. Table 2 shows the glomerular hemodynamic parameters obtained for group 2 (acute FK). This evaluation was not performed for acute infusion of FK vehicle, since whole kidney function was not altered by this treatment (shown in Table 1). FK provoked a significant ($P < 0.05$) 40% decline in single nephron GFR (SNGFR) and nonsignificant reductions in Q_A (25%) and K_f (22%). Total arteriolar resistance (R_T) was only slightly increased (13%) and no significant alterations were observed in glomerular or tubular hydraulic pressures.

In the chronic FK group (Table 3), reductions in GFR (23%, $P < 0.05$, compared with FK vehicle) and RPF (29%, $P < 0.05$ vs. vehicle) were observed. TRVR increased slightly (32%, $P < 0.05$) and FF remained stable. The micropuncture studies (Table 4) showed that chronic FK induced a decline in SNGFR (23%, $P < 0.05$), in Q_A (38%, $P < 0.05$) and in K_f (41%, $P < 0.05$), compared with vehicle. The reduction in Q_A occurred as a result of an

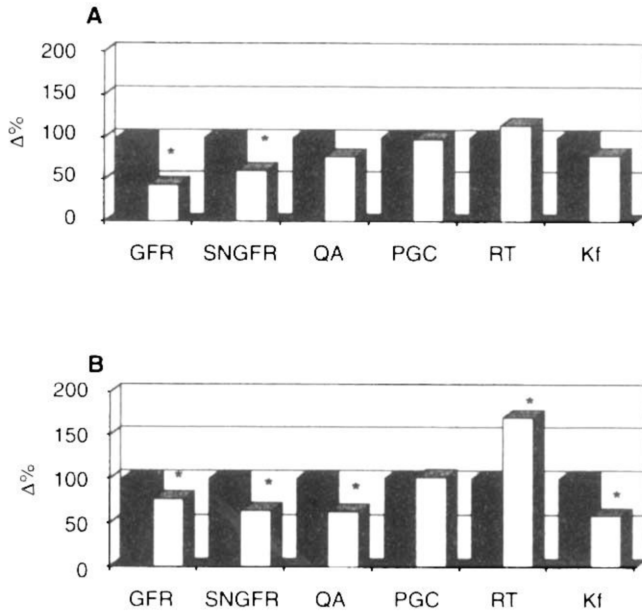

Fig. 1. *Comparative (Δ%) hemodynamic effects before (pre, ■) and after (after, □) acute and chronic FK 506 administration.* *$P < 0.05$ vs. pre-FK.

elevation in both arteriolar resistances, R_A (55%, $P < 0.05$) and R_E (100%, $P < 0.05$). No effect of chronic FK was observed on mean glomerular hydraulic pressure (P_{GC}) or on tubular hydraulic pressure (P_T).

Morphological studies using light microscopy revealed no alteration in the glomeruli, tubules, vessels or interstitial compartment.

Data obtained for intracellular calcium were expressed as the mean of the 340/380 ratios since this index presents a lower variability than the concentration in molarity and thus provides more reliable results. Figures 2 to 7 were constructed on basis of this ratio, and the results are also calculated in molarity and presented in the text.

When the action of FK on MC was studied, it was observed that FK caused increases in $[Ca^{2+}]_i$ in all experiments in group 1 (R 340/380: 2.5 ± 0.3 vs. 3.8 ± 0.4, $N = 10$, $P < 0.02$). A typical experiment is shown in Figure 2, where $[Ca^{2+}]_i$ varied from 85 nM (basal) to 109 nM (in the presence of 10^{-6} M of FK). However, these $[Ca^{2+}]_i$ increments were blunted when a channel blocker (verapamil) was used (Group 2, R 340/380, 3.5 ± 0.9 to 2.8 ± 0.8), as shown in Figure 3 (261 nM vs. 249 nM). When the MC suspension was studied in the Ca^{2+}-free buffer, the levels of $[Ca^{2+}]_i$ were maintained after FK administration (Group 3, R 340/380, 1.5 ± 0.3 vs. 1.4 ± 0.3). However, when $CaCl_2$ was added to the extracellular medium, an increase in $[Ca^{2+}]_i$ (R 340/380: 1.4 ± 0.3 to 2.2 ± 0.5, $P < 0.05$) was observed without an additional FK dose. Figure 4 shows a typical experiment with a basal $[Ca^{2+}]_i$ of 3 nM, reaching 41 nM after the addition of $CaCl_2$ (final bath concentration of 1.36 mM). The $[Ca^{2+}]_i$ did not change even when additional 1.36 mM $CaCl_2$ was added to the bath (final concentration of 2.7 mM). This observation suggests that FK acts on the cellular membrane and the increase of $[Ca^{2+}]_i$ depends on the presence of extracellular Ca^{2+}. To determine if the effects of FK on $[Ca^{2+}]_i$ are related to Ca^{2+}-release-activated channels (CRAC), we used thapsigargin (TSG), an inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum (ER) membrane [27]. Figure 5 exemplifies an experiment using thapsigargin. The addition of TSG provoked a slow and progressive increase in fluorescence, reaching a maximal value (phase "a" from 60 nM to 124 nM) (Group 4, R 340/380, 1.7 ± 0.03 to 2.2 ± 0.15). Phase "a" was followed by a decrease in fluorescence without returning to baseline (phase "b" $[Ca^{2+}]_i$, 80 nM; and R 340/380, 2.2 ± 0.15 to 2.1 ± 0.12) as a consequence of an influx of Ca^{2+} via CRAC. This influx probably was stimulated by the depletion of Ca^{2+} from ER, as suggested by Takemura et al [27], observed in different cells. After this phase, the maximal dose of FK was given (10^{-6} M; phase "c"), and a progressive and slow decrease in the intensity of fluorescence was observed in the direction of baseline values ($[Ca^{2+}]_i$, 77 nM; R 340/380, basal × FK 1.70 ± 0.03 vs. 2.13 ± 0.09, $P < 0.05$), suggesting that FK was not able to activate additional channels. Figure 6 shows that TSG in the absence of extracellular Ca^{2+} caused a progressive and slow increment in fluorescence, but of lower intensity when compared with the experiment with normal extracellular calcium concentration (Fig. 5). The addition of $CaCl_2$ to the solution (1.36 mM) caused an increase of the initial value (from 38 nM to 112 nM), and a further increment (to 148 nM) was observed when an additional dose of Ca^{2+} was added to the solution (final concentration of 2.7 mM). The later addition of 10^{-6} M FK caused a further increment in $[Ca^{2+}]_i$ (to 184 nM), suggesting that FK acts on channels other than CRAC. Figure 7 shows a protocol similar to that illustrated in Figure 6, except in the presence of verapamil. In the presence of thapsigargin the maximum value of 121 nM was reached. With the addition of calcium to the bath, first to a concentration of 1.36 mM and later to double this value (2.7 mM), the $[Ca^{2+}]_i$ changed from 91 nM to 252 nM and 314 nM. Verapamil had no blocking effect on CRAC, but inhibited the FK effect, since when it was added, the $[Ca^{2+}]_i$ decreased to 250 nM. In this situation, after addition of extracellular calcium, an increase in $[Ca^{2+}]_i$ due to a calcium influx via CRAC was observed and, when FK was added, it was not able to further increase $[Ca^{2+}]_i$. These data suggest the dependence of the action of FK on the voltage-dependent channels in MC. Therefore, our results suggest that FK increases $[Ca^{2+}]_i$ by acting on voltage-dependent channels without stimulating CRAC. Additionally, FK was able to cause contraction of MC (Fig. 8) by a 50% reduction in cross-sectional area (CSA) from 3772 ± 106 to 1912 ± 61 μm^2 ($P < 0.05$).

Discussion

FK is a liposoluble macrolide antibiotic, isolated from the fermentation broth of a soil fungus named *Streptomyces tsukubaiensis* [28]. Like CsA, it inhibits T cell activation and lymphokine production *in vitro* and suppresses both cell-mediated immunity and allograft rejection in experimental animals [29, 30]. FK has been widely used as an immunosuppressor in human transplantation since 1989 [31], but a number of side effects have been described, with nephrotoxicity being the main undesirable effect whose cause has not been completely determined [1, 2, 7, 32, 33].

In the present study, the acute administration of FK provoked an important fall in total glomerular filtration rate (GFR) due to a decrease in total renal plasma flow rate (RPF), consequent to the increase in mean total vascular renal resistance (TRVR). The micropuncture studies showed that the reduction of 40% in single nephron glomerular filtration rate (SNGFR) was the result of a combination of a 25% decline in glomerular plasma flow rate (Q_A) and a 22% decline in glomerular ultrafiltration coefficient

Table 2. Glomerular hemodynamic parameters in the acute FK group

Group	SNGFR	Q_A	SNFF	R_A	R_E	R_T	P_{GC}	P_T	ΔP	π_E	K_f	Eq/Dis
	nl/min		*%*	$\times 10^{10} \cdot dyn \cdot sec \cdot cm^{-5}$			*mm Hg*				*nl/s · mm Hg*	
Acute FK (N = 9)												
before	43.0	133.4	37	2.2	1.3	3.5	42	12	30	30	0.1009	6/3
	± 5.2	± 9.8	± 1	± 0.4	± 0.4	± 0.3	± 1	± 1	± 1	± 1	± 0.0203	
after	26.0[a]	99.8	28[a]	2.6	1.5	4.1	41	12	29	29	0.0790	4/5
	± 2.5	± 12.0	± 1	± 0.4	± 0.3	± 0.8	± 1	± 1	± 1	± 1	± 0.0130	

Data are X ± SEM. Abbreviations are: Eq, equililibrium; Dis, disequilibrium.
[a] $P < 0.05$ after vs. before

Table 3. Summary of general parameters and whole kidney function in the chronic groups: Vehicle (VEH) and FK

Groups	Body wt in	Body wt fn	Kidney wt	MAP	Hct	$\dot{V}$	GFR	RPF	FF	TRVR
	g			*mm Hg*	*%*	*μl/min*	*ml/min*		*%*	*mm Hg · min/ml*
Chronic VEH	242	254	0.83	99	45	4.8	0.97	2.80	38	20.3
(N = 8)	± 11	± 11	± 0.03	± 1	± 1	± 1.0	± 0.02	± 0.30	± 1	± 2.0
Chronic FK	220	220	0.78	92[a]	41[a]	4.1	0.75[a]	1.98[a]	38	26.8[a]
(N = 8)	± 4	± 6	± 0.04	± 2	± 2	± 0.6	± 0.04	± 0.11	± 1	± 1.7

Data are X ± SEM. Abbreviations are: in, initial; fn, final.
[a] $P < 0.05$ FK vs. VEH

Table 4. Glomerular hemodynamic parameters during chronic administration of Vehicle (VEH) or FK

Group	SNGFR	Q_A	SNFF	R_A	R_E	R_T	P_{GC}	P_T	ΔP	π_E	K_f	Eq/Dis
	nl/min		*%*	$\times 10^{10} \cdot dyn \cdot sec \cdot cm^{-5}$			*mm Hg*				*nl/sec · mm Hg*	
Chronic VEH	37.9	117.6	32	2.2	0.9	3.1	42	17	25	29	0.1486	6/2
(N = 8)	± 3.0	± 10.2	± 1	± 0.1	± 0.1	± 0.2	± 1	± 1	± 1	± 1	± 0.0101	
Chronic FK	29.1[a]	73.5[a]	40[a]	3.4[a]	1.8[a]	5.2[a]	43	17	26	31	0.0870[a]	6/2
(N = 8)	± 1.9	± 6.1	± 1	± 0.4	± 0.2	± 0.5	± 2	± 1	± 2	± 1	± 0.0110	

Data are X ± SEM. Abbreviations are: Eq, equilibrium; Dis, disequilibrium.
[a] $P < 0.05$ FK vs. VEH

(K_f), since the transglomerular hydraulic pressure difference (ΔP) was unchanged (Fig. 1). The reduction in Q_A was due to a slight (17%) increase in total arteriolar resistance (R_T). Although the decline in K_f was not significant, one should consider that a larger number of animals were in filtration pressure equilibrium during the control period, before FK (6/3), allowing the calculation of only a minimum K_f. This ratio was shifted to 4/5 after FK administration, that is, more than 50% of animals reached the disequilibrium filtration pressure, permitting the calculation of unique K_f values. Thus, in the acute group, during the control period the calculated K_f could be lower than the actual value.

Despite these quantitative differences, the glomerular hemodynamic pattern induced by FK is similar after acute or chronic treatment. In contrast, in our laboratory we have observed distinct patterns for acute and chronic CsA treatment [4, 5]. Acutely, CsA at a dose of 50 mg/kg i.v. caused a decrease in Q_A by increasing R_A but also caused an impressive increase in R_E, thus elevating P_{GC} (Fig. 9). The acute effect of CsA caused a decrease in K_f. In contrast, short-term (9 days) chronic CsA administration caused a preferential increase in R_A without a change in K_f [5], as also previously observed by Thompson et al [34]. Differently from FK and CsA, acyclovir showed a distinct hemodynamic pattern, as shown in Figure 9. Acutely the decrease in SNGFR was due to

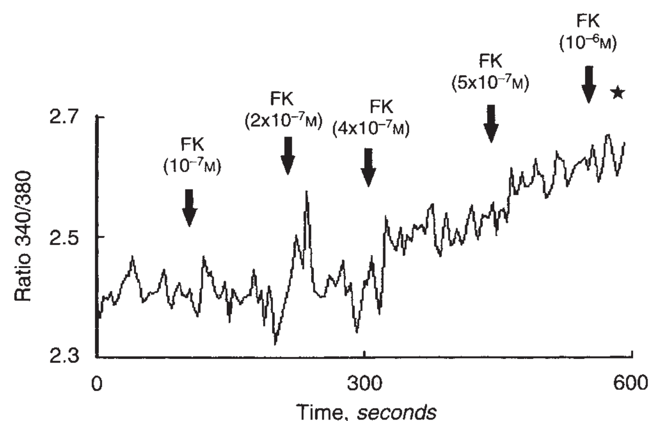

Fig. 2. *Typical experiment showing the effect of progressive and cumulative doses of FK 506 on $[Ca^{2+}]_i$ in mesangial cells in culture.* *$P < 0.02$ vs. baseline.

reductions in Q_A and in P_{GC}, with no changes in K_f [10]. However, chronic administration of acyclovir caused a hemodynamic pattern similar to that induced by FK chronic treatment [36, 37]. Comparative analyses of the hemodynamic patterns induced by

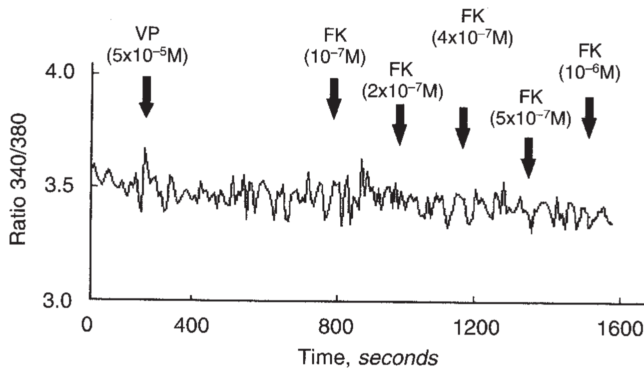

Fig. 3. *Typical experiment showing the effect of cumulative doses of FK 506 on $[Ca^{2+}]_i$ in mesangial cells previously incubated with verapamil (VP).*

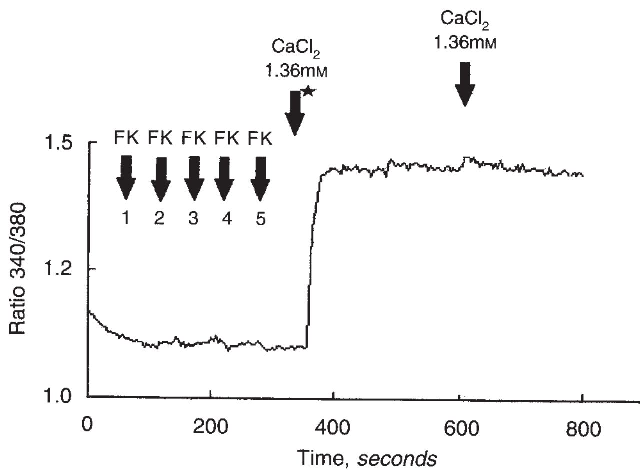

Fig. 4. *Typical experiment showing the effect of cumulative doses of FK on $[Ca^{2+}]_i$ in mesangial cells in Ca^{2+}-free buffer and later addition of $CaCl_2$ to the solution.* FK doses are: (1) 10^{-7} M; (2) 2×10^{-7} M; (3) 4×10^{-7} M; (4) 5×10^{-7} M; (5) 10^{-6} M. *$P < 0.05$ vs. FK.

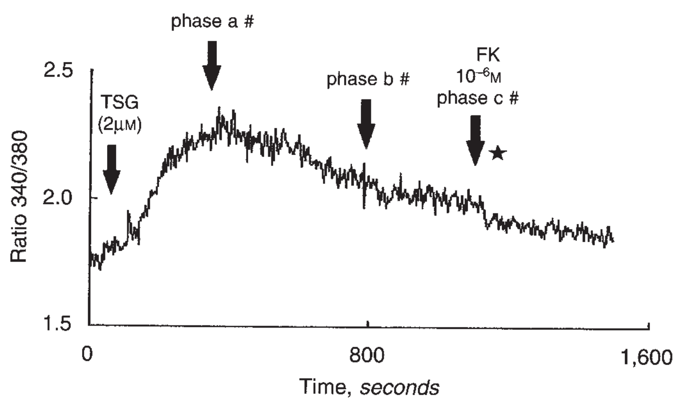

Fig. 5. *Typical experiment showing the maximal effect of FK 506 (10^{-6} M) on $[Ca^{2+}]_i$ in mesangial cells after incubation with thapsigargin (TSG).* *$P <$ 0.05 vs. baseline. #Phases a, b, and c are in the text.

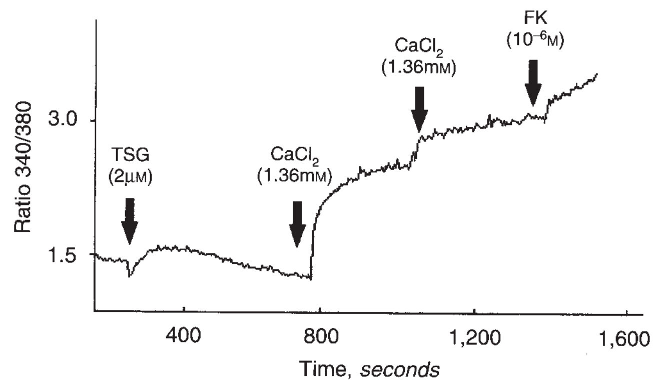

Fig. 6. *Effect of the maximal FK 506 dose (10^{-6} M) on $[Ca^{2+}]_i$ in mesangial cells in Ca^{2+}-free buffer after thapsigargin (TSG) and later addition of two successive doses of $CaCl_2$ (1.36 mM).*

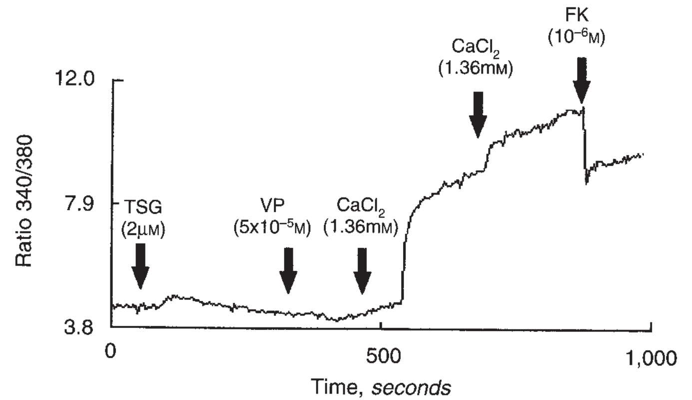

Fig. 7. *Effect of the maximal dose of FK 506 (10^{-6} M) on $[Ca^{2+}]_i$ in mesangial cells in Ca^{2+}-free buffer after incubation with thapsigargin (TSG), verapamil (VP) and two successive doses of $CaCl_2$ (1.36 mM).*

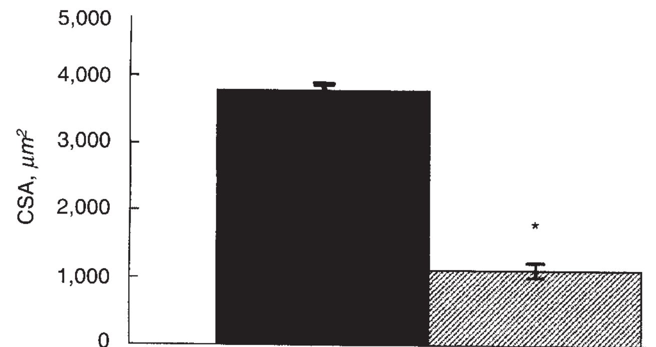

Fig. 8. *Morphometry (cross sectional area-CSA) of mesangial cells in control and after incubation with a maximal FK 506 dose (10^{-6} M).* Symbols are: (■) control, $N = 112$; (▨) FK 506, $N = 99$. *$P < 0.05$ vs. control.

these three drugs suggest that they affect the renal microcirculation in different ways, although the common final result is a reduction in SNGFR. Additionally, these modifications are also dependent on acute or chronic administration. The patterns presented in Figure 9 show that CsA and acyclovir provoke different profiles in the microcirculation if they are administered acutely or chronically, inducing changes in the afferent and efferent arteriolar resistances that result in distinct modifications in the glomerular hydraulic pressures, with or without changes in K_f. Another interesting observation is that the hemodynamic profile may be qualitatively similar under acute and chronic

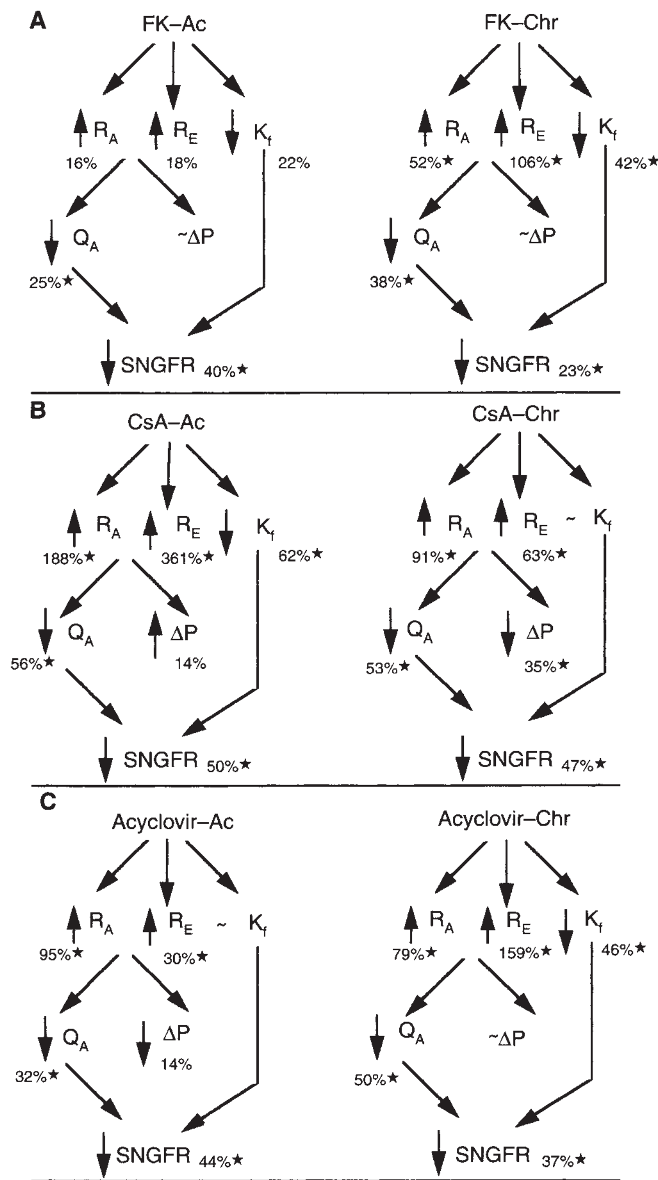

Fig. 9. *Comparative glomerular hemodynamic patterns after acute (Ac) and chronic (Chr) administration of (**A**) FK 506, (**B**) cyclosporine (CsA) and (**C**) acyclovir.* Reference data are from Csa [4, 5] and acyclovir [10, 35]; data are $\bar{X} \pm$ SEM; $^*P < 0.05$.

administration, as observed for FK (Fig. 9). Therefore, a comparative evaluation of acute and chronic hemodynamic patterns needs to be done in order to better understand the sequential events in the renal microcirculation. Furthermore, the observation of different acute and chronic hemodynamics may suggest the best pharmacological intervention in order to minimize nephrotoxicity, by choosing drugs that preferentially affect afferent or efferent arterioles.

The reduction in K_f mainly observed after chronic FK administration, 42% (chronic) versus 22% (acute), suggests that mesangial cells may participate in this nephrotoxicity by reducing K_f (Fig. 9). To further evaluate the role of mesangial cells in this mechanism, $[Ca^{2+}]_i$ was determined and morphometric analyzes of MC area were performed after FK treatment.

Calcium plays an important role as a transmembrane messenger in the lymphocyte activation signal. Initial observations on the relationship between extracellular Ca^{+2} and lymphocyte activation demonstrated that early removal of Ca^{+2} from the medium decreased lymphocyte transformation [35–38]. Additional evidence for the Ca^{2+} dependence on the initial activation signal was observed during studies demonstrating that mitogenic lectins fail to stimulate lymphocyte proliferation in Ca^{2+}-free medium [38, 39]. Moreover, the importance of changes in $[Ca^{2+}]_i$ altering mesangial surface area is well known and thus we decided to evaluate the potential effect of FK on changes in $[Ca^{2+}]_i$.

The effect of FK on the MC caused increases in $[Ca^{2+}]_i$ since the fluorescence intensity of the complex Ca^{2+}-sensitive dye Fura-2 is directly related to the increment of $[Ca^{2+}]_i$ [24]. The data presented in this study are expressed as the 340/380 ratio since the determination of calcium concentration using the Grynkiewicz's formula involves, for instance, the dissociation constant (K_d) of Ca^{2+} from Fura-2, which is known to be strongly influenced by the absolute viscosity of the cytoplasm of the different cell types [40]. However, in order to provide an example of $[Ca^{2+}]_i$ concentration in molarity, values used in the representative Figures were expressed in molarity, obtained by the formula of Grynkiewicz, Poenie and Tsien [24]. It was observed that the increases in $[Ca^{2+}]_i$ were proportional to the increases of the FK doses (Fig. 2). It is well known that the increase of $[Ca^{2+}]_i$ is due to intracellular pool mobilization via second messenger [41] or via Ca^{2+} influx from the extracellular medium by membrane voltage-dependent channels or by calcium release activating channels (CRAC) stimulated by depletion of Ca^{2+} from ER [27]. Typical experiments illustrated in Figures 3 and 4 show that FK was unable to increase $[Ca^{2+}]_i$ after verapamil incubation or during the absence of extracellular calcium in the buffer. These results suggest that the increase in $[Ca^{2+}]_i$ is dependent on extracellular calcium influx via stimulation of type L voltage-dependent channels in the membrane. On the other hand, when extracellular calcium concentration was normalized, FK significantly increased the intensity of fluorescence (Fig. 4). However, part of this increment could be due to the liberation of Ca^{2+} from intracellular pools, especially from the ER.

Takemura et al [27], using TSG, a blocker of Ca^{2+}-ATPase from the ER, caused a progressive and slow depletion of Ca^{2+} in the ER. This event caused an increase in fluorescence intensity until a plateau was reached, as shown in Figure 5. After awhile, with the reduction of Ca^{2+} from ER, a stimulus to the cytoplasm membrane, via ionic current or via a soluble factor (CIF) [42] induces an opening of the CRAC, thus leading to an influx of extracellular calcium. This phenomenon was observed in our results during the second phase shown in Figure 5, that is, the sustained phase. Therefore, the capacitative model of Takemura et al [27] is present in the MC. Also, after activation of CRAC, FK was unable to further increase $[Ca^{2+}]_i$, suggesting that this drug could not open additional channels after a striking $[Ca^{2+}]_i$ increase consequent to CRAC stimulation. Figure 6 shows that MC, when incubated in the absence of calcium in the buffer, could not present the sustained phase with TSG, since this phase is dependent on the influx of calcium via CRAC. With the addition of extracellular Ca^{2+} a quick increment of $[Ca^{2+}]_i$ was observed, suggesting a stimulus to the cytoplasm membrane from the ER. In

this situation, FK induced an additional increase in $[Ca^{2+}]_i$, indicating its action on membrane channels other than CRAC. This possibility was confirmed in protocol 6 when MC were incubated in the absence of Ca^{2+}, treated with TSG and later incubated with verapamil. $[Ca^{2+}]_i$ was stable but increased when the extracellular calcium concentration was replenished, demonstrating the CRAC effect. The addition of FK in the presence of TSG and verapamil blunted any further increment in $[Ca^{2+}]_i$, thus confirming the hypothesis that FK acts on the voltage-dependent channels that were blocked by verapamil (Fig. 7).

The increase in $[Ca^{2+}]_i$ leads to many cellular functions, including cellular contraction [41]. Using morphometric analyzes, we observed a reduction in MC surface area (Fig. 8), suggesting that FK caused contraction of MC. Thus, it is reasonable to suggest the occurrence of a reduction of the surface area of glomerular ultrafiltration (reduction in K_f), in agreement with the observations of glomerular hemodynamics *in vivo*. Similar observations were made by Kumano et al [43] using Diltiazem®, a calcium channel blocker that was able to blunt the effects of FK on the hemodynamics of rats acutely treated with FK. The present data suggest that the contraction of MC potentially participates in FK nephrotoxicity *in vivo*.

Nephrotoxicity is a complex phenomenon consisting of various mechanisms including a variety of hormonal systems participating in this pathophysiology that cause difficult treatment. However, sequential studies, time- and dose-dependent studies as well as cellular protocol studies that allow the evaluation of independent pathways can contribute to a better understanding of nephrotoxicity and perhaps to a more rational treatment. The nephrotoxicity of FK may be prevented or minimized since it depends on extracellular Ca^{2+} mobilization, and thus Ca^{2+} blockers could interfere with the hemodynamic effects and also contribute to prevention of the rejection process due to their immunosuppressor activity [44].

Acknowledgments

We thank Dr. Seymour Mong, Fujisawa Pharmaceutical Company, for kindly supplying FK 506. We also thank Dr. Euthimia Brandão Almeida Prado, Faculdade de Medicina da Universidade de São Paulo, for the mesangial cell immunofluorescence staining. This work was supported by grants from FAPESP, FINEP, CNPq, CAPES and IPEPENHI. Partial data were published (*Transplant Proc* 24:3082, 1992) and presented as abstracts at the Annual American Society of Nephrology Meeting (1991), XIIth International Congress of Nephrology (1993), International Satellite Symposium on Acute Renal Failure-Greece (1993), and XV Congresso Brasileiro de Nefrologia (1994).

Reprint requests to Nestor Schor, M.D., Ph.D., Nephrology Division, Department of Medicine, Escola Paulista de Medicina, Rua Botucatú 740, 04023-062 São Paulo, SP Brasil.

References

1. Demetris AJ, Banner B, Fung J, Jordan M, Starzl TE: Histopathology of human renal allograft rejection under FK 506: A comparison with cyclosporine. *Transplant Proc* 23:944–946, 1991
2. Shapiro R, Jordan M, Fung J, McCauley J, Johnston J, Iwaki Y, Tzakis A, Hakala T, Todo S, Starzl TE: Kidney transplantation under FK 506 immunosuppression. *Transplant Proc* 23:920–923, 1993
3. Nalesnik MA, Todo S, Murase N, Gryzan S, Lee P-H, Makowka L, Starzl TE: Toxicology of FK-506 in the Lewis rat. *Transplant Proc* 19:89–92, 1987
4. Barros EJG, Boim A, Santos FPH, Ramos OL, Schor N: Glomerular hemodynamics and hormonal participation on cyclosporine nephrotoxicity. *Kidney Int* 32:19–25, 1987
5. Barros EJG, Boim MA, Santos OFP, Moura LAR, Ramos OL, Schor N: Short-term chronic effects of cyclosporine on glomerular hemodynamics in rats. *J Nephrol* 4:227–233, 1990
6. Mené P, Simonson S, Dunn MJ: Physiology of the mesangial cell. *Physiol Rev* 69:1347–1424, 1989
7. Japanese FK 506 Study Group: Morphological characteristics of renal allografts showing renal dysfunction under FK 506 therapy: Is graft biopsy available to reveal the morphological findings corresponding with FK 506 nephrophaty? *Transplant Proc* 25:624–630, 1993
8. Liu JD, Farmer J, Lane WS, Weissman I, Schreiber SL: Calcineurin is a common target of cyclophilin-cyclosporine A and FKBP-FK 506 complexes. *Cell* 606:807–810, 1991
9. Foor F, Stephan AP, Morin N, Dahl AM, Ramadam N, Charbit G, Bostian AK, Nielsen BJ: Calcineurin mediates inhibition by FK-506 and cyclosporine of recovery from alpha factor arrest in yeast. *Nature* 360:682–684, 1992
10. Santos MFF, Boim MA, Santos OFP, Ajzen H, Schor N: Experimental acute renal failure by acyclovir in rats: Glomerular hemodynamics evaluation. *JASN* 4:758, 1993
11. Schor N, Ichikawa I, Renke HG, Troy JL, Brenner BM: Pathophysiology of altered glomerular function in aminoglycoside-treated rats. *Kidney Int* 19:288–296, 1981
12. Santos OFP, Boim MA, Bregman R, Barros EJG, Schor N: Role of platelet activating factor in gentamicin nephrotoxicity. *Kidney Int* 40:742–747, 1991
13. Kon V, Sugiura M, Inagami T, Harvie BR, Ichikawa I, Hoover RL: Role of endothelin in cyclosporine-induced glomerular dysfunction. *Kidney Int* 37:1487–1491, 1990
14. Boim MA, Ajzen H, Ramos OL, Schor N: Glomerular hemodynamics and hormonal evaluation during starvation in rats. *Kidney Int* 42:567–572, 1992
15. Maddox DA, Price DC, Rector FC Jr: Effects of surgery on plasma volume and salt and water excretion in rats. *Am J Physiol* 233:F600–F606, 1977
16. Denn WM, Robertson CR, Brenner BM: A model of glomerular ultrafiltration in the rat. *Am J Physiol* 223:1178–1183, 1972
17. Denn WM, Troy JL, Robertson CR, Brenner BM: Dynamics of glomerular ultrafiltration in the rat: IV. Determination of the ultrafiltration coefficient. *J Clin Invest* 52:1500–1508, 1973
18. Fuhr J, Kaczmarczyk J, Kruttgen CD: Eine einfache colorimetrische Methode zur Inulin Bestimmung for Nierenclearance Utersuchegen bei Soffwechsel gesunder and diabetibern. *Klin Wochenschr* 33:729–730, 1955
19. Smith HW, Finkeltein N, Aliminosa L, Crawford B, Graber M: The renal clearances of substitute hippuric acid derivatives and other aromatic acids in dog and man. *J Clin Invest* 24:388–404, 1945
20. Vurek GG, Pegram SE: Fluorimetric method for the determination of nanogram quantities of inulin. *Anal Biochem* 16:409–419, 1966
21. Viets JW, Deen HM, Troy JL, Brenner BM: Determination of serum protein concentration in nanoliter blood samples using fluorescamine or ophthalaldehyde. *Anal Biochem* 88:513–21, 1978
22. Schimdt-Nielsen B, Barret JM, Graves B, Crossley B: Physiological and morphological responses of the rat kidney to reduced dietary protein. *Am J Physiol* 248:F31–F42, 1985
23. Greenspon SA, Krakower CA: Direct evidence for the antigenicity of the glomeruli in the production of nephrotoxic serums. *Archiv Pathol* 49:291–297, 1950
24. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450, 1985
25. Rodrígues-Puyol D, Lamas S, Oliveira A, López-Farré A, Ortega G, Hernando L, López-Novoa JM: Actions of cyclosporine A on cultured rat mesangial cell. *Kidney Int* 35:632–637, 1989
26. Hollander M, Wolfe DA: *Non parametric statistical methods*. New York, John Wiley and Sons, 1973, pp 503
27. Takemura H, Hughes AR, Thastrup O, Putney W Jr: Activation of calcium entry by tumor promoter thapsigargin in parotid acinar cells. *J Biol Chem* 264:12266–12271, 1989

28. GOTO T, KINO T, NISHIYAMA M, OKUHARA M, KOHSAKA M, AOKI H, IMANAKA H: Discovery of FK-506, a novel immunosuppressant isolated from Streptomyces tsukubaiensis. *Transplant Proc* 19:4–8, 1987
29. KINO T, HATANAKA H, MIYATA S, INAMURA N, NISHIYAMA M, YAJIMA T, GOTO T, OKUHARA M, KOHSAKA M, AOKI H, OCHIAI T: FK-506, A novel immunosuppressant isolated from Streptomyces. II. Immunosuppressive effect of FK-506 in vitro. *J Antibiot* 40:1256–1265, 1987
30. OCHIAI T, NAKAJIMA K, NAGATA M, SUZUKI T, ASANO T, UEMATSU T, GOTO T, HORII S, KENMOCHI T, NAKAGOORI T, ISONO K: Effect of a new immunosuppressive agent, FK 506, on heterotopic cardiac allotransplantation in the rat. *Transplant Proc* 24:1284–1286, 1987
31. STARZL TE, TODO S, FUNG J, DEMETRIS AJ, VENKATARAMMAN R, JAIN A: FK 506 for liver, and pancreas transplantation. *Lancet* 2:1000–1004, 1989
32. FUNG GJJ, ALLESSIANI M, ABU-EELMAGOD K, IODO S, SHAPIRO R, TZAKIS A, THIEL DV, ARMITAGE J, JAIN A, MCCAULEY JJ, SEEBY R, STARZL TE: Adverse effects associated with the use of FK-506. *Transplant Proc* 23:3105–3107, 1991
33. STARZL TE: FK 506 versus cyclosporine. *Transplant Proc* 25:511–512, 1993
34. THOMPSON SC, TUCKER BJ, GABBAI F, BLANTZ RC: Functional effects on glomerular hemodynamics of short-term chronic cyclosporine in male rats. *J Clin Invest* 83:960–969, 1989
35. SANTOS MFF, SANTOS OFP, BOIM MA, RAZVICKAS CV, AJZEN H, SCHOR N: Acyclovir (ACV) and Gancyclovir (Gan) administration in rats: Glomerular hemodynamics evaluation. (abstract) *JASN* 5:931, 1994
36. KAY JE: Interaction of lymphocytes and phytohemagglutinin inhibition by chelating agents. *Exp Cell Res* 68:11–18, 1971
37. WHITNEY RB, SUTHERLAND RM: Requirement of calcium ions in lymphocyte transformation stimulated by phytohemoagglutinin. *J Cell Physiol* 80:329–337, 1972
38. MAINO VC, GRENN NM, CRUMPTON MJ: The role of calcium ions in initiating transformation of lymphocytes. *Nature* 251:324–327, 1974
39. FREEDMAN MH, KHAN NR, TREW-MARSHALL BJ, CUPPLES CG, MÉLY-GOUBERT B: Early biochemical events in lymphocyte activation. *Cell Immunol* 58:134–146, 1981
40. WILLIAMS DA, FAY RS: Intracellular calibration of the fluorescent calcium indicator Fura-2. *Cell Calcium* 11:75–83, 1990
41. CARAFOLI E: Intracellular calcium homeostasis. *Ann Rev Biochem* 56:395–433, 1987
42. FASOLATO C, INNOCENTI B, POZZAN T: Receptor-activated Ca^{2+} influx: How many mechanisms for how many channels. *TIPS* 15:77–83, 1994
43. KUMANO K, WANG G, ENDO T, KUWAO S: FK 506-induced nephrotoxicity in rats. *Transplant Proc* 23:512–515, 1991
44. DUMONT L, CHEN H, DALOZE P, DALOZE P, DASHENG X, GARCEAU D: Immunosuppressive properties of the benzothiazepine calcium antagonists diltiazem and clentiazem, with and without cyclosporine, in heterotopic rat heart transplantation. *Transplantation* 56:181–184, 1993