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FK506 mechanism of nephrotoxicity: stimulatory effect on endothelin secretion by cultured kidney cells

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Abstract. The administration of FK506 or cyclosporin A (CyA) to animals and humans induces a decrease in glomerular filtration rate and renal plasma flow and an increase in renal vascular resistance. Endothelins (ET-1), very powerful renal vasoconstrictors, are involved in CyA-related alteration in renal haemodynamics. In this study we sought to determine whether FK506 and CyA had a stimulatory effect on endothelin secretion by cultured kidney cells (tubular and mesangial cells) and whether this stimulatory effect coincided with an increase in ET-1 serum level in FK506- and CyA-treated rats. FK506 concentrations of 1, 0.1, 0.01 and 0.001 μM significantly stimulated ET-1 secretion by cultured tubular and mesangial cells. CyA at 10, 1, 0.1 and 0.01 μM also exerted an enhancing effect on ET-1 secretion in cultured tubular cells whereas CyA only at 10 and 1 μM had a stimulatory effect on ET-1 secretion by human mesangial cells. We observed that the concentrations of CyA that induced the most substantial enhancing effect were 10 or 100 times higher than those required for FK506 to produce the same effect. The concentrations of FK506 or CyA which induced ET-1 secretion by tubular cells and kidney cells were not cytolytic as assessed by N-acetyl-β-D-glucosaminidase (NAG) release and lactic dehydrogenase (LDH) release. FK506 or CyA treatment at toxic doses induced an increase in serum level of ET-1 in treated rats. We conclude that FK506 and CyA induced an increase in the synthesis of endothelin in the kidney which may explain the increase in circulating ET-1. This stimulatory effect may contribute to the genesis of haemodynamic preturbations associated with FK506 and CyA.

Key words: FK506 – Cyclosporine – Endothelin – Mesangial cell – Kidney tubular cell

FK506 is a newly developed immunosuppressive drug which has been used successfully in clinical transplantation [4, 13]. Among its recognized side effects, nephrotoxicity is the most prominent [3]. Its molecular structure is unrelated to cyclosporin A (CyA), but both agents have similar pharmacological effects and may possibly have the same toxicity. It has been reported that FK506 decreases the glomerular filtration rate (GFR) and renal plasma flow (RFP) and increases renal vascular resistance in humans [19] and in rats [7] treated with FK506. These reversible haemodynamic perturbations that have also been observed in CyA nephrotoxicity, have been linked to the renin angiotensin, adrenergic systems, and thromboxane A2 [8, 11]. Endothelin (ET), a peptide isolated from supernatants of cultured porcine aortic endothelial cells is a very powerful renal vasoconstrictor [20]. ET is ten times more potent than angiotensin II, vasopressin, or neuropeptide Y, making it the most potent endogenous vasoactive substance known [20]. ET appears to have a pivotal role in the pathophysiology of CyA-induced acute renal vasoconstriction and glomerular dysfunction [5]. Renal cell lines and mesangial cells physiologically express mRNA for endothelin and secrete this peptide in the supernatant [14, 21]. In this study we demonstrated that FK506 and CyA have a stimulatory effect on endothelin secretion by kidney cells and this effect coincided with a significant rise in the serum level of ET-1 in FK506- and CyA-treated rats. This finding may contribute to the clarification of the mechanism of FK506- and CyA-induced vasoconstriction and glomerular dysfunction.

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Materials and methods

Cell isolation and culture

The LLC-PK1 tubular cell line was used in the present study. This porcine kidney tubular cell line has the characteristics of renal proximal tubular cells [2]. Tubular cells were cultured in medium M199 (Nissui Pharmaceutical Ltd., Tokyo, Japan) with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Utah, USA). Cell subcultures were obtained by incubating a washed confluent tubular cells culture with a solution of 0.25% trypsin and 0.02% EDTA (Gibco) for 5 min at 37 °C.

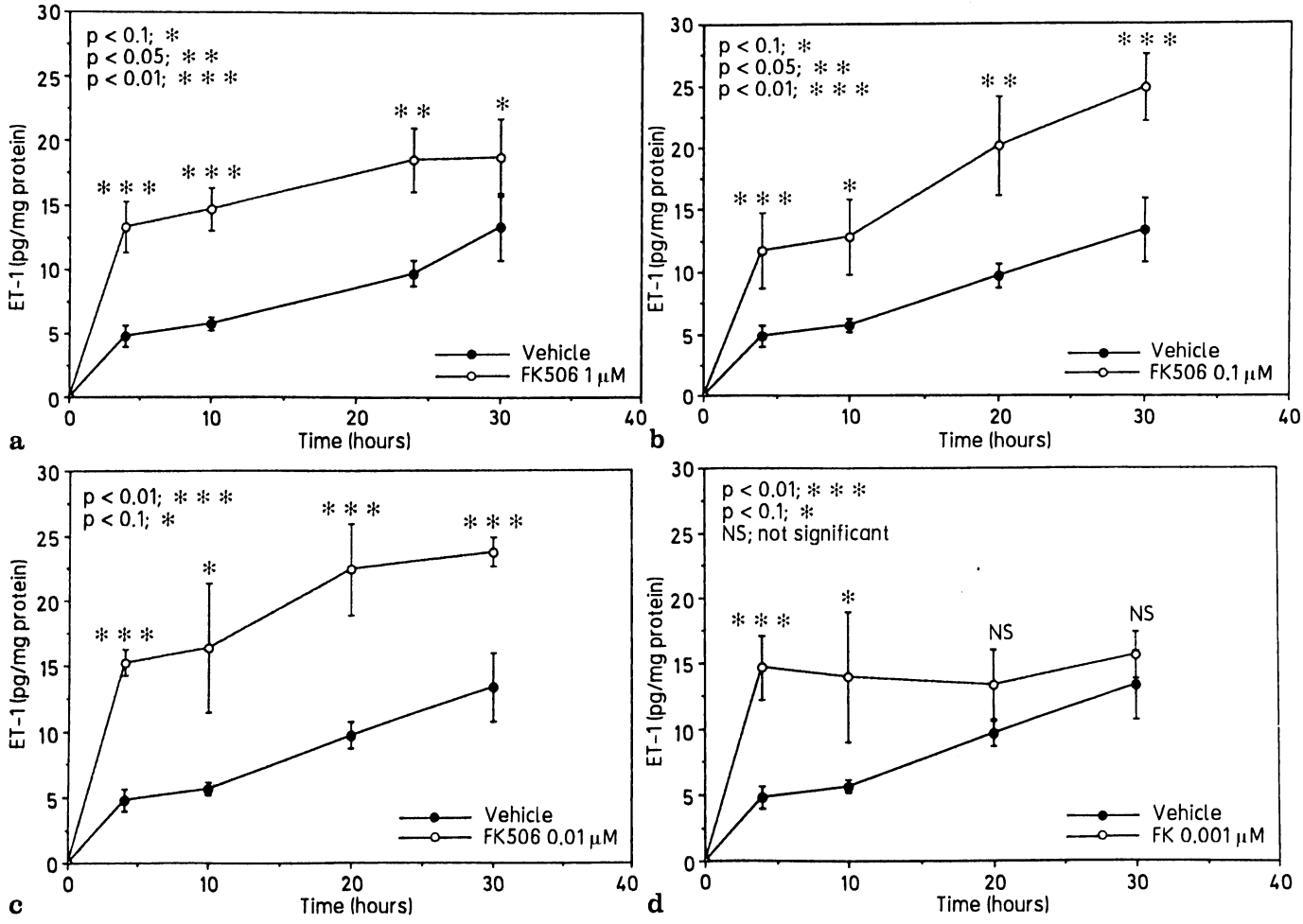

Fig. 1. Kinetics of endothelin production by cultured human tubular cells treated with FK506. Monolayers of tubular cells were incubated with medium containing FK506 at concentrations of 1, 0.1, 0.01 and 0.001 μ*M*. The vehicle alone served as control. The supernatants were harvested at 4, 10, 20 and 30 h and assayed for ET-1

The culture of human mesangial cells (HMC) was performed as previously described [17]. Briefly, glomeruli were isolated from pieces of nephrectomised kidneys and searched for renal cell carcinoma by a consecutive sieving method. Then the glomeruli were seeded onto 10-cm culture dishes (Becton Dickinson, Oxnard, Calif.) and cultured at 37°C in an atmosphere of 95% air and 5% CO_2 using a tissue culture medium RPMI-1640 supplemented with 20% FBS. HMC between the third passage and the fourth passage were used in this study.

Drug preparations

FK506 (Fujisawa, Osaka, Japan) was dissolved in absolute methanol. CyA (Sandoz, Basel, Switzerland) was dissolved in absolute ethanol before being added to the media.

Animal drug administration

Male Lewis rats weighing 100–120 g were used. FK506 dissolved in 5% arabic gum solution was administered by gastric gavage at a dose of 5 mg/kg per day. CyA also dissolved in arabic gum was administered by the same procedure at a dose of 20 mg/kg per day. The control rats received the vehicle alone. Each group consisted of five rats. After 12 days administration, the rats' sera were analysed for ET-1.

Sandwich enzyme immunoassay (EIA) for the detection of ET-1

Since human, porcine and rat ET-1 are identical [20], this assay can be used for detection of ET-1 from the three species. After reaching confluence, the tubular cells were fed with M199 10% FBS and HMC with RPMI 20% FBS, containing various concentrations of FK506 or CyA. The final methanol or ethanol concentration for all concentrations of FK506 or CyA and vehicle was 0.2%. The media were collected at 4, 10, 24, 30, 48 and 72 h and analysed for ET-1. The EIA was performed as descibed previously [16]. Briefly, mouse anti-ET-1 monoclonal antibody-coated microtest plates were prepared by adding 100 μl of the monoclonal antibody AwETN40 (20 μg/ml) (kindly provided by Dr. N. Suzuki, Tsukuba Research Laboratory, Takeda Chemical Industries, Japan) to each well, followed by the addition of 300 μl of Block Ace (Snow Brand Milk Product Co., Japan) diluted four times with PBS. ET-1 standards or samples to be tested in 100 μl buffer D (0.02 *M* phosphate buffer, pH 7, containing 10% Block Ace, 0.4 *M* NaCl, and 2 m*M* EDTA) were added to each well and incubated at 4°C for 1 day. After being washed with PBS, the plate was reacted with 100 μl anti-endothelin Fab'-horseradish peroxidase (provided by Dr. N. Suzuki) at a dilution of 1/400 in buffer C (buffer containing 50 μ*M* 0.02 *M* phosphate buffer, pH 7, 1% bovine serum albumin, with 0.4 *M* NaCl and 2 m*M* EDTA) at 4°C for 1 day. After washing with PBS the bound enzyme activity was measured using *o*-phenylenediamine as chromogen. The data were analysed by expressing ET-1 production on the basis of total amount of cell protein.

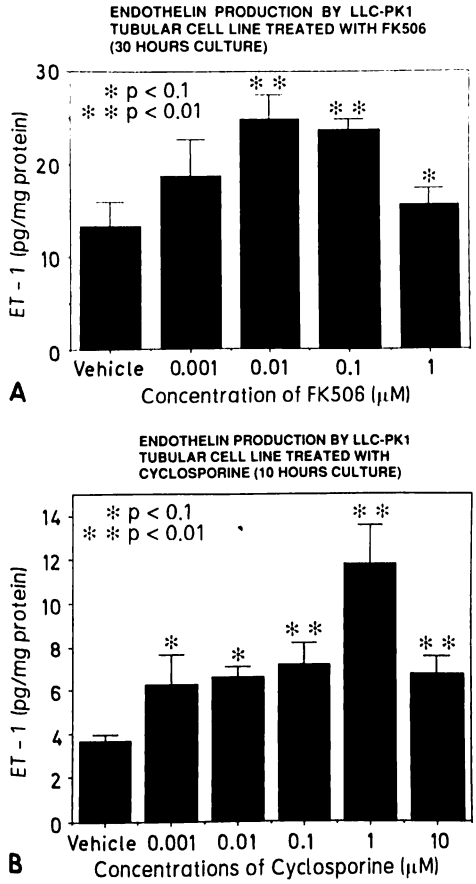

Fig. 2 A. Endothelin production by LLC-PK1 tubular cell line treated with FK506 for 30 h culture. **B** Endothelin production by LLC-PK1 tubular cell line treated with CyA for 10 h

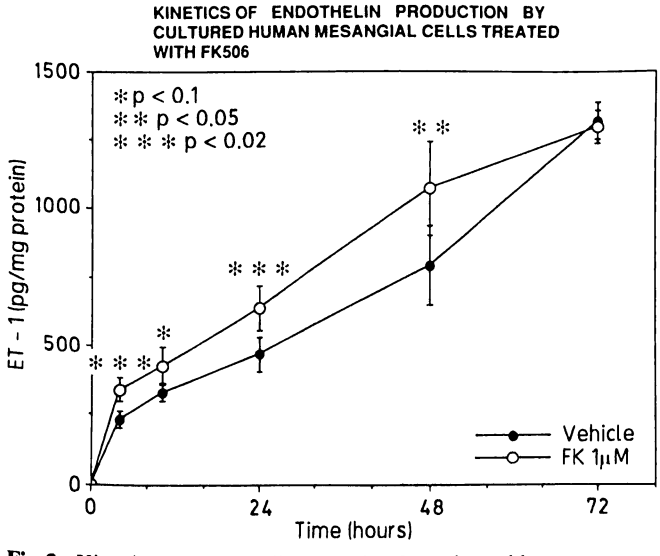

Fig. 3. Kinetics of endothelin secretion by cultured human mesangial cells treated with 1 μ*M* FK506

N-Acetyl-β-D-glucosaminidase (NAG) determination

The supernatants from monolayer of tubular cells treated with different concentrations of FK506, CyA or appropriate concentration of vehicle for 24 h were assayed for NAG. NAG was determined using *m*-cresolsulphonphtaeinyl-N-β-glucosaminidase method (Shionogi NAG assay kit, Shionogi, Osaka, Japan).

Lactate dehydrogenase (LDH) assay

The supernatants harvested from FK506- or CyA-treated HMC at 4, 10, 24 and 48 h were assayed for LDH release by a spectrophotometric assay using commercial kits (Sigma). Maximum release was determined by freezing and thawing the cells three times. Spontaneous release was determined by incubation in medium alone. The results were expressed as percentage of specific release: (LDH test − LDH spontaneous) × 100/(LDH freeze and thaw − LDH spontaneous).

Results

Effect of FK506 and cyclosporine on ET-1 production by LLC-PK1 tubular cell line and human mesangial cells

ET-1 secretion by tubular cells cultured in the absence or presence of FK506 is shown in Fig. 1. Incubation of confluent monolayer tubular cells with culture medium containing vehicle alone resulted in basal production which increased with the duration of incubation. The generation of ET-1 by tubular cells incubated with medium alone was not different from that observed with medium and vehicle (data not shown). FK506 at concentrations of 1, 0.1, 0.01 μ*M* significantly stimulated ET-1 productin in cultured tubular cells. This stimulatory effect was seen at 4, 10, 20 and 30 h incubations. Low concentrations of FK506 (0.001 μ*M*) enhanced ET-1 secretion by tubular cells only with 4 and 10 h incubations. The most substantial enhancing effect of FK506 was observed with concentrations of 0.1 and 0.01 μ*M* (Fig. 1 and 2 A). CyA at concentrations of 10, 1, 0.1 and 0.01 μ*M* also significantly increased ET-1 production by tubular cells (Fig. 2 B). The most marked stimulatory effect was observed with 1 μ*M* CyA. Therefore, it appears that the CyA concentration that induced the maximal enhancing effect was 10 or 100 times higher than the concentration of FK506 that produced the same effect.

As shown in Fig. 3, HMC in vitro secreted more ET-1 than tubular cells (about 50 times). The incubation of HMC with medium and vehicle was not different from that with medium alone. FK506 at 1 μ*M* significantly enhanced ET-1 secretion by cultured HMC with 4, 10, 24 and 48 h of incubation (Fig. 3). FK506 concentrations 0.1, 0.01 and 0.001 also significantly stimulated ET-1 production with a substantial enhancing effect at 0.1 μ*M* FK506 (Fig. 4 A). CyA at 1 and 10 μ*M* had a substantial enhancing effect on ET-1 secretion by HMC whereas concentrations below 1 μ*M* were without significant stimulatory effect (Fig. 4 B). In HMC the maximal stimulatory effect on ET-1 secretion was also obtained with CyA concentrations 10 or 100 times higher than the concentration of FK506 that produced the same effect.

Circulating level of ET-1 in FK506- and cyclosporine-treated rats

After 12 days of oral administration of FK506 or CyA, the rats became sick and lost 30% of their weight. In control rats ET-1 was not detectable in our assay system (less than 1.6 pg/ml). In FK506-treated rats, ET-1 level was 17.7 ± 5.7 pg/ml. In CyA-treated rats, ET-1 level was 18 ± 5.9 pg/ml.

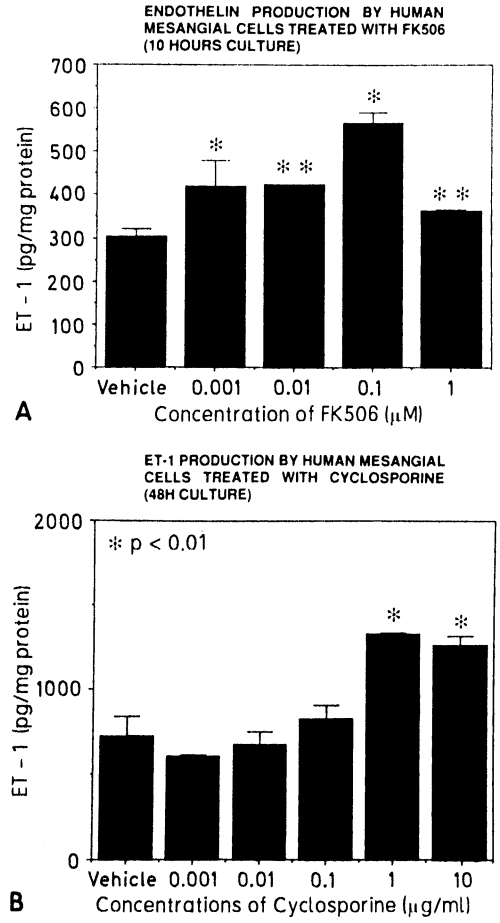

Fig. 4. A Endothelin production by cultured human mesangial cells treated with FK506 for 10 h culture. **B** Endothelin production by human mesangial cells treated with cyclosporine for 48 h

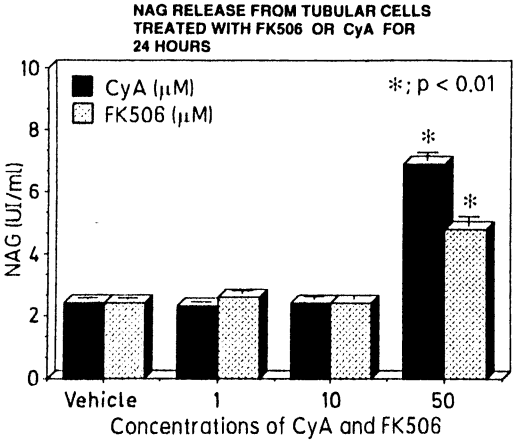

Fig. 5. NAG release from tubular cells treated with FK506 or CyA for 24 h. Supernatants from tubular cells treated with various concentrations of FK506 or CyA were assayed for NAG as descibed in Materials and methods. The vehicle methanol or ethanol served as control

FK506 and cyclosporine NAG release from tubular cells

NAG release was used as a marker of tubular cell injury. NAG release from tubular cells incubated with FK506 doses of 10 μ*M* and 1 μ*M* for 24 h was not different from that of the control (Fig. 5). Furthermore, the kinetics of NAG release (at 4, 10, 24 and 48 h) from tubular cells treated in vitro with FK506 or 10, 1, 0.1 or 0.01 μ*M* CyA were very similar to those of the vehicle alone (unpublished data). In contrast, high doses of FK506 and CyA (50 μ*M*) induced a significant release of NAG from treated tubular cells ($P < 0.01$).

Effect of FK506 on LDH release from HMC

The cytolytic effect of FK506 and CyA on HMC was assayed using LDH release. HMC treated with FK506 or CyA concentrations 10 and 1 μ*M* for 4, 10, 24 and 48 h showed almost no LDH release (0.1–0.2 %). In contrast, with high concentrations of FK506 (50 μ*M*), we observed a relatively high LDH release (Table 1).

Discussion

Extensive investigations have been performed to clarify the mechanism by which CyA and recently FK506 induce tubular and vascular injury which appears to be the primary target in FK506- and CyA-induced damage [5]. In the present study we have demonstrated that FK506 and CyA, at non-cytolytic concentrations, stimulates ET-1 secretion by cultured kidney cells. This effect coincides with an increase in serum level of ET-1 in FK506- and CyA-treated rats.

Renal tubular cell lines synthesise and release ET-1 [14] as is confirmed in this study. ET-1 secretion by tubular cells is stimulated by exposure to a variety of agents such as thrombin, IL-1β, TNF, and TGFβ [10]. FK506 and CyA also stimulated the secretion of ET by tubular cells. Since FK506 and CyA at the range of concentrations tested for ET secretion were not cytolytic (Fig. 6), it is unlikely that the increase in ET secretion in the media could be attributed to cell leakage or release from dying cells. The secretion may therefore be the result of active cellular secretion. The most important enhancing effect on ET-1 secretion by tubular cells was observed with CyA concentrations 10 or 100 times higher than those required for FK506 to produce the same effect. This finding is concordant with the comparative pharmacological effect of both drugs. It has been established that FK506 is 10 or 100 times more immunosuppressive than CyA [18]. Therefore, the present finding lends further support to the relationship between immunosuppressive activity and toxicity.

Human mesangial cells constitutively secrete ET-1 in culture media [21]. The stimulation of HMC by inflammatory mediators such thrombin, thromboxane A2, TGFβ [21] or endotoxin [15] resulted in an increase in ET-1 secretion. HMC, which regulate glomerular filtration, responded to FK506 or CyA treatment in vitro by an increase in their ability to secrete ET-1. This secretion seemed to be an active secretion from cells, since no LDH release (Table 1) was observed at the range of concentration of FK506 or CyA used to induce ET-1 secretion.

The rats' serum levels of endothelin after 12 days oral administration of FK506 or CyA were elevated as compared with values in controls. Kon et al. [5] also reported an elevation in ET-1 serum level 20 min after intravenous administration of CyA in rats. The pathophysiological significance of the stimulatory effect of FK506 and CyA on

Table 1. LDH release from human mesangial cells treated with FK506 or cyclosporine. Supernatants from monolayers of HMC treated with different concentrations of FK506 or CyA were harvested at 4, 10, 24 and 48 h and were assayed for LDH using a spectrophotometric method. The vehicle alone served as control, maximal release was obtained by three cycles of freezing and thawing. The data are expressed as percentage of specific release

	LDH specific release (%) Incubation time (h)			
	4	10	24	48
FK506 1 μM	0.10	0.20	0.10	0.15
FK506 10 μM	0.15	0.10	0.12	0.10
FK506 50 μM	1.80	1.30	9.50	17.00
CyA 1 μM	0.10	0.12	0.10	0.13
CyA 10 μM	0.20	0.1	0.11	0.12
CyA 50 μM	ND	ND	ND	ND

ND, not determined

ET secretion by kidney cells remains to be defined, but as suggested by Kon et al. [5] the increased level of ET-1 following CyA and FK506 may reflect a perturbed ET synthesis and/or metabolism in the kidney after FK506 or CyA treatment, which causes overflow of locally produced ET into the circulation, and changes in tissue and receptor characteristics. Recently, it was demonstrated that CyA-induced glomerular dysfunction involves upregulation of glomerular ET receptors and that this is specific to the kidney [9]. If the effect of ET on glomerular haemodynamics [6] is compared with the effect reported with CyA [1], the two are similar. In isolated perfused kidney exposed to specific anti-endothelin antibody, but not immunized rabbit serum, the CyA-induced fall in RPF and GFR was markedly reduced [12]. The role of endogenous ET in acute CyA nephrotoxicity has been documented in vivo by studies showing that the infusion of specific ET serum partially but significantly prevented the reduction of GFR and RPF induced by intravenous administration of CyA [5]. Since there are only a few reports available concerning the effect of the new drug FK506 on mesangial and tubular cell physiology, this study may provide the experimental background to understanding the glomerular dysfunction which has been reported with FK506 administration [7, 19]. We conclude that the stimulatory effect of FK506 on ET-1 secretion by kidney cells may contribute to the genesis of FK506- and CyA-induced glomerular dysfunction.

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