

Renal Endothelin in FK506-Induced Nephrotoxicity in Spontaneously Hypertensive Rats

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ABSTRACT—FK506, a major immunosuppressive agent, often causes nephrotoxicity accompanied by renal vasoconstriction. It is recognized that endothelin (ET) plays a role in the cyclosporin A-induced nephrotoxicity, but the involvement of ET in the FK506-induced renal dysfunction is still poorly understood. We elicited nephrotoxicity by daily administration of FK506 in spontaneously hypertensive rats, and we examined the renal gene expression of ET and its receptors and the effects of an ET receptor antagonist on FK506-induced renal dysfunction. FK506 administration (4 mg/kg/day, i.m.) for 14 days induced nephrotoxicity, including a renal vasoconstriction and a decrease in glomerular filtration rate. The renal dysfunction was accompanied by an increase in ET-1 mRNA levels, while ET_B-receptor mRNA was unaffected. Continuous administration of an ET_A/ET_B antagonist, TAK-044 (3 mg/day, s.c.), which effectively blocked systemic and renal vascular responses to exogenously administered ET-1, partially attenuated the FK506-induced renal vasoconstriction. However the reduced glomerular filtration rate were not affected by TAK-044. Thus, although enhanced gene expression of ET-1 in the kidney is involved in the renal vasoconstriction, ET does not play a major role in the FK506-induced renal dysfunction.

Keywords: FK506, Endothelin, Nephrotoxicity, TAK-044, Spontaneously hypertensive rats

FK506 and cyclosporin A (CsA) are potent immunosuppressive agents and have been successfully used for the treatment of allograft rejection. Although these drugs are structurally unrelated, their mechanisms of immunosuppression share the common characteristic that they both inhibit the function of calcineurin and thus lower the interleukin (IL)-2 gene expression in T lymphocytes (1, 2). Nephrotoxicity is a common feature of their adverse effects, which includes renal vasoconstriction, at least in the acute phase (3). Clinical studies indicate that FK506 differs from CsA in that it causes less systemic vasoconstriction and hypertension, whereas both of them induce a similar renal vasoconstriction (3).

A number of endogenous substances have been proposed to mediate the renal dysfunction by CsA. Several lines of evidence suggest that endothelins (ETs) play an important role. In vitro studies with isolated afferent arterioles and isolated perfused kidney support the role of endothelin on CsA-induced vasoconstriction (4–6). In the in vivo situation, acute intravenous ad-

ministration of CsA to anesthetized rats markedly elevated plasma concentration of ET-1 and induced renal vasoconstriction and a decrease in glomerular filtration rate (GFR), which were blocked by either an ET antibody or an ET-receptor antagonist (7–10). Although additional mechanisms may be involved in the renal vasoconstrictor action of CsA (5, 10, 11), daily administration of CsA to rats for several days to weeks resulted in an increase in plasma creatinine and a decrease in creatinine clearances (Ccr), which were attenuated by ET_A-selective or ET_A/ET_B-non-selective antagonists (12–15). ET-receptor antagonists also attenuated the hypertension during CsA administration (16, 17). CsA and its metabolites consistently increase ET-1 release from cultured endothelial cells or mesangial cells (18–20). Thus, it is clear that ET plays an important role in the CsA-induced renal dysfunction and hypertension.

In contrast, the role of ET in the FK506-induced renal dysfunction is still unclear. In some studies, FK506 increased the production of ET in vitro (21–23), but in other studies, it had no effects on ET release (24, 25). Furthermore, no in vivo studies elucidating the role of ET

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in the FK506-induced renal dysfunction have been reported. This is, at least in part, due to the lack of good animal models for the renal dysfunction by FK506. Hammond and Kind mentioned that the FK506-induced renal dysfunction, as measured by standard parameters (e.g., plasma creatinine increase), is not readily observed in most rat strains tested, presumably due to their large reservoir of renal function (26).

The purpose of the present study is to elucidate the role of ET in FK506-induced renal dysfunction. We used spontaneously hypertensive rats (SHR) that are one of a few strains susceptible to FK506-induced nephrotoxicity (27). We examined the renal mRNA levels of ET and receptors and the effects of an ET_A/ET_B-non selective ET-receptor antagonist, TAK-044 (28), on the FK506-induced renal dysfunction in SHR.

MATERIALS AND METHODS

Animals

Experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male SHR (Charles River, Inc. Japan, Tokyo) (15–17 weeks, 290–345 g) maintained on standard commercial rat chow and tap water ad libitum were used, unless otherwise stated.

Chemicals

FK506 formulae were gifts from Fujisawa Pharmaceutical Co., Ltd., Osaka. Each vial of FK506 for intramuscular injection containing 20 mg of tacrolimus hydrate, 4 mg of polyoxyethylene hydrogenated oil and 50 mg of D-mannitol was suspended with physiological saline before injection. FK506 for intravenous injection (Prograf Injection) containing 5 mg of tacrolimus hydrate, 200 mg of polyoxyethylene hydrogenated oil and 0.6 g of ethanol was diluted with physiological saline before intravenous infusion. TAK-044, [cyclo D- α -aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L- α -aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl]disodium salt, was a gift from Takeda Chemical Industries Ltd. (Osaka) and was dissolved in physiological saline.

Preliminary studies to characterize FK506-induced nephrotoxicity

Immediate renal hemodynamic responses to FK506 in anesthetized rats: To confirm the absence of immediate renal hemodynamic responses to FK506 (25, 29), eight male Sprague-Dawley (SD) rats weighing 300–350 g were anesthetized with thiopental sodium (100 mg/kg, i.p.). The rectal temperature was controlled at 37–37.5°C by a heating blanket and a lamp (ATB-1100; Nihon Kohden, Osaka). The trachea was cannulated to facilitate spon-

taneous breathing. The right femoral artery and vein were cannulated for direct blood pressure (BP) measurement and drug or saline (9 ml/kg/hr) infusion, respectively. The left kidney was exposed through a flank incision and a flow probe (FI-010T, ϕ 1 mm; Nihon Kohden) was placed around left renal artery. Renal blood flow (RBF) was continuously measured by an electromagnetic flow meter (MFV-2100, Nihon Kohden). Sixty minutes after surgery, FK506 was infused intravenously to 5 rats at a dose of 5 mg/kg in 10 min. Three rats were administered vehicle instead of FK506. Following FK506 administration, observation was continued for 1 hr.

Effects of daily administration of FK506 to SHR: Ten SHR were randomly assigned into 2 groups. Before the start of experiments, systolic BP was determined by a tail-cuff method. FK506 (4 mg/kg/day) or vehicle was administered intramuscularly once daily for 14 days (FK506 treated group, n=5; vehicle treated group, n=5). On the 14th day following the last drug administration, rats were placed in an individual metabolic cage for 24-hr urine collection. During the metabolic cage study, rats were fasted with free access to tap water. At the end of the study, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and blood samples were drawn from the abdominal aorta and collected into precooled tubes containing 2Na-EDTA and aprotinin (at a final blood concentration of 2.7 mM and 500 KIU/ml, respectively). Thereafter, kidneys were rapidly excised, placed in liquid nitrogen and then stored at –80°C until used for RNA extraction (FK506 treated group, n=4; vehicle treated group, n=4) and measurement of renal ET-1 contents (FK506 treated group, n=5; vehicle treated group, n=5). Plasma and urine samples were stored at –20°C until assay for plasma ET-1, blood urea nitrogen (BUN) and creatinine concentration.

Effects of chronic blockade of ET receptor on FK506-induced nephrotoxicity in SHR

To elucidate the role of renal ET on FK506-induced renal dysfunction, animals were continuously administered an ET_A/ET_B-receptor antagonist, TAK-044, and the effects on FK506-induced nephrotoxicity were examined. SHR were randomly assigned into 4 groups. All rats were implanted subcutaneously with Alzet mini-osmotic pumps filled with either TAK-044 or saline at the back under ether anesthesia. Six hours after implantation, intramuscular injection of FK506 or vehicle was started once daily for 14 days. Group 1 rats received vehicle and saline. Group 2 rats received vehicle and TAK-044 (3 mg/day, ca 10 mg/kg/day, s.c.). Group 3 rats received FK506 (4 mg/kg/day, i.m.) and saline. Group 4 rats receive FK506 and TAK-044

Metabolic cage study: On the 7th day following blood

sampling from the tail vein, rats in each group were housed in individual metabolic cages for 24-hr urine collection. On the 14th day, 24-hr urine collection was again performed. At the end of the study, rats were anesthetized with ether and a polyethylene catheter was introduced into the right femoral artery. After blood sampling (ca 300 μ l), the catheter was exteriorized through a cutaneous tunnel at the back of the neck. Six hours after surgery, BP was measured by a pressure transducer (100T, Nihon Kohden) under a conscious condition (group 1, n=7; group 2, n=9; group 3, n=9; group 4, n=9).

Renal hemodynamic study: In a separate set of experiments, animals were divided into 4 groups as described above. Twenty-four hours after the last injection of FK506 on the 14th day, animals in each group were anesthetized with thiopental sodium (100 mg/kg, i.p.) and were surgically prepared for RBF measurement as described above. In addition, urinary bladder was cannulated for timed urine collection. After surgery, 1 ml of physiological saline containing 6 g/dl bovine serum albumin (BSA) (Fraction V; Pentex, Bayer, IL, USA) was injected intravenously in order to compensate for the loss of protein. Then, intravenous infusion of saline containing inulin was started at a rate of 9 ml/kg/hr (inulin: 100 mg/kg plus 180 mg/kg/hr). Sixty-90 min after the surgery, urine was collected for 30 min and a blood sample was drawn into capillary tubes at the midpoint of the urine collection period (group 1, n=6; group 2, n=5; group 3, n=6; group 4, n=6) (30). The renal vascular resistance (RVR) was calculated by dividing BP by RBF. GFR was estimated by the clearance of inulin. After the clearance study, kidneys from group 1 (n=3) and 3 (n=3) were excised to examine the effects of FK506 on mRNA levels in the renal cortex and medulla. While cooling, the outer cortex and medulla were rapidly dissected, frozen and stored at -80°C until RNA extraction.

Effectiveness of chronic ET receptor blockade: To assess the effectiveness of ET receptor blockade with TAK-044, ET-1 (Peptide Institute, Inc., Osaka) dissolved in saline (0.1% BSA) was intravenously injected into rats from group 1 (n=5) and group 2 (n=4), including those used in renal hemodynamic study. Each rat received three bolus injections of ET-1 (0.1, 0.3 and 1.0 nmol/kg, 1 ml/kg) at 30- to 60-min intervals when BP and RBF reverted toward the control levels and reached plateaus.

24-hr trough concentration of FK506: In a separate experiment, 24 hr after the last injection of FK506, blood was obtained from the aorta of rats (groups 3 and 4, n=5, respectively) under anesthesia with pentobarbital sodium (50 mg/kg, i.p.).

Analyses

Plasma and urinary creatinine concentrations, urinary *N*-acetyl β -glucosaminidase (NAG) and urine γ -glutamyltranspeptidase (γ -GTP) were measured by standard laboratory techniques. Na and K concentrations in plasma and urine were determined by flame photometry. Plasma and urinary inulin was determined by the methods of Walser et al. (31). Plasma renin activity (PRA) was determined by incubating with an equal volume of 250 mM Na phosphate buffer (pH 7.0) containing 5 mM EDTA and 4 mM phenylmethylsulfonyl fluoride at 37°C . Angiotensin I generated during a 1-hr incubation was determined by radioimmunoassay. FK506 concentrations in the whole blood were determined by ELISA with a monoclonal anti-tacrolimus antibody. To measure the renal tissue content of ET-1, frozen kidneys were homogenized with 10 parts of 1 M acetate and 0.1% Triton X (polytron mixer 60 sec) and were immediately heated at 100°C for 10 min. After chilling, the homogenate was centrifuged at $25,000 \times g$ for 30 min, and the supernatant was removed and stored. ET-1 concentrations in plasma and renal tissue were measured by a sandwich enzyme immunoassay kit (Immuno-Biological Lab., Inc., Fujioka) following SepPak C18 extraction. The detection limit of the assay is 0.78 pg/ml, and the cross-reactivity to ET-3 and big ET-1 is less than 0.1%.

cDNA probes

cDNA probes used were as follows: rat preproET-1 cDNA (2.0-kb *EcoRI-EcoRI* fragment) (32), a gift from Dr. T. Sakurai, Tsukuba University; rat ET_A cDNA (1.5-kb *Sac I-Xba I* fragment) and rat ET_B cDNA (1.334-kb *EcoRI-EcoRI* fragment) (33), gifts from Prof. S. Nakanishi, Kyoto University; rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1.3-kb *Pst I-Pst I* fragment) (34).

Extraction of total RNA and Northern blot analysis

All procedures were performed as previously described (35). In brief, total RNA was extracted from whole kidney, renal cortex and medulla by the guanidium thiocyanate-phenol-chloroform method with minor modification. Twenty micrograms of total RNA was electrophoresed on 1% agarose gel and transferred to a nylon membrane (Gene Screen Plus; EI du Pont de Nemours & Co., NEN Products, Boston, MA, USA). The membrane was hybridized with the above mentioned ^{32}P -dCTP-labeled cDNA probes, then washed and finally exposed to the imaging plate (BAS-III; Fuji Photo film Co., Tokyo). To evaluate tissue mRNA levels, autoradiograms were digitized to measure density, using a bioimaging analyzer (BAS-2000, Fuji Photo film Co.). The density of an individual mRNA band was divided by that of GAPDH

mRNA, to correct for differences in RNA loading and transfer to a nylon membrane.

Data analyses

Data are expressed as means \pm S.E. Comparisons between 2 groups were made by using either Student's *t*-test or Mann-Whitney *U*-test where appropriate. To examine the effects of ET antagonist on FK506-induced events, data were analyzed by ANOVA, and individual comparisons were made by the Student-Newman-Keuls test. A *P* value smaller than 0.05 was considered statistically significant.

RESULTS

Acute renal effect of FK506 in SD rats

With acute intravenous administration of vehicle for FK506, BP transiently increased and RBF increased with a concomitant decrease in calculated RVR. The hemodynamic changes were essentially same as those seen following FK-506 administration. No renal vasoconstriction was observed during the hour after FK506 administration at a dose of 5 mg/kg (data not shown).

Table 1. Effects of a 2-week treatment of SHR with FK506 on renal and tubular function and ET-1 concentration in plasma and renal tissue

	Vehicle n=5	FK506 n=5
BUN (mg/dl)	18.9 \pm 2.9	50.5 \pm 3.7*
Pcr (mg/dl)	0.44 \pm 0.02	0.78 \pm 0.04*
PNa (mEq/l)	144 \pm 1.0	143 \pm 0.8
PK (mEq/l)	4.1 \pm 0.3	4.3 \pm 0.1
Ccr (ml/min/100 gbw)	0.43 \pm 0.03	0.27 \pm 0.02*
Urinary NAG (U/day)	0.19 \pm 0.03	0.15 \pm 0.01
Urinary γ -GTP (mIU/day)	9 \pm 4 (n=4)	15 \pm 1 (n=4)
Plasma ET-1 (pg/ml)	0.63 \pm 0.19	0.54 \pm 0.16 (n=4)
Renal ET-1 content (pg/g)	175 \pm 14	219 \pm 18

Values are expressed as means \pm S.E. Rats were each housed in an individual metabolic cage following FK506 treatment for 2 weeks. BUN, blood urea nitrogen; Pcr, plasma creatinine concentration; Ccr, creatinine clearance. **P* < 0.05, vs vehicle.

Induction of nephrotoxicity in SHR by FK506

Daily administration of FK506 (4 mg/kg/day) for 2 weeks significantly increased BUN and plasma creatinine concentrations. Endogenous Ccr was significantly decreased in FK506-treated rats. In contrast, total urinary

Table 2. Effects of TAK-044 on FK506-induced alterations in systemic and renal functional parameters obtained from metabolic cage study

	FK506 TAK-044 Group	(-) Vehicle		4 mg/kg/day for 14 days	
		(-) n=7 1	(+) n=9 2	(-) n=9 3	(+) n=9 4
Body weight (g)	pre	322 \pm 7	319 \pm 5	331 \pm 4	324 \pm 3
	7 day	325 \pm 5	336 \pm 4	302 \pm 6*	299 \pm 3*
	14 day	330 \pm 6	334 \pm 4	287 \pm 6*	298 \pm 4*
MBP (mmHg)	15 day	171 \pm 2	163 \pm 2 [#]	153 \pm 1*	150 \pm 1*
Heart rate (/min)	15 day	406 \pm 16	394 \pm 7	410 \pm 7	423 \pm 10
BUN (mg/dl)	7 day	26.7 \pm 1.0	23.4 \pm 1.0	34.4 \pm 2.3*	37.5 \pm 1.1*
	15 day	25.7 \pm 2.2	20.0 \pm 1.3	59.8 \pm 2.6*	65.0 \pm 1.5*
Pcr (mg/dl)	7 day	0.63 \pm 0.02	0.57 \pm 0.02	0.70 \pm 0.05*	0.77 \pm 0.04*
	15 day	0.60 \pm 0.05	0.60 \pm 0.02	0.86 \pm 0.04*	0.79 \pm 0.06*
Ccr (ml/min/100 g bw)	7 day	0.35 \pm 0.02	0.38 \pm 0.02	0.28 \pm 0.04*	0.30 \pm 0.02*
	15 day	0.36 \pm 0.01	0.39 \pm 0.02	0.24 \pm 0.02*	0.29 \pm 0.02*
FENa (%)	7 day	0.12 \pm 0.02	0.14 \pm 0.02	0.14 \pm 0.02	0.14 \pm 0.02
	15 day	0.13 \pm 0.02	0.11 \pm 0.01	0.24 \pm 0.02*	0.21 \pm 0.03*
FEK (%)	15 day	10.4 \pm 1.3	8.5 \pm 0.5	16.5 \pm 2.3*	13.9 \pm 0.9*

Values are expressed as means \pm S.E. Rats were each housed in an individual metabolic cage at 7 and 14 days after the start of FK506 administration. Blood pressure and heart rate were measured under the conscious condition directly through an arterial catheter placed at the end of the study. MBP, mean blood pressure; BUN, blood urea nitrogen; Pcr, plasma creatinine concentration; Ccr, creatinine clearance; FENa, fractional excretion of Na; FEK, fractional excretion of K. **P* < 0.05, compared to the corresponding group without FK506. [#]*P* < 0.05, compared to the corresponding group without TAK-044.

Table 3. Effects of TAK-044 on PRA and 24-hr trough FK506 whole blood levels following 2 weeks of FK506 administration

FK506 TAK-044 Group	(-) Vehicle		4 mg/kg/day for 14 days	
	(-)	(+)	(-)	(+)
	1	2	3	4
PRA (ngAI/ml/hr)	22.6±3.5	29.8±4.9	60.9±11.3*	54.0±4.0*
FK506 in whole blood (ng/ml)			20.8±1.2	24.4±2.9

Values are expressed as means±S.E. *P<0.05, compared to the corresponding group without FK506. PRA, plasma renin activity; AI, angiotensin I.

excretion of NAG and γ -GTP was unchanged with FK506 treatment. Although plasma ET-1 concentration was not affected by FK506, the renal ET-1 contents in the FK506-treated group tended to be higher than those in vehicle-treated rats, but this difference was not statistically significant ($P=0.078$) (Table 1). Therefore, we used the daily administration of FK506 (4 mg/kg/day, 2 weeks) for the following experiments.

Effect of TAK-044 on the FK506-induced nephrotoxicity

Metabolic cage study: Table 2 summarizes data obtained from the metabolic cage study that examined the effect of TAK-044 on FK506-induced nephrotoxicity. Although the body weight in vehicle-treated rats remained unchanged, rats treated with FK506 for 2 weeks lost about 10% of their weight. Systolic BP before treatment was the same among the groups (data not shown). Following completion of the treatments, mean BP (MBP) determined under the conscious condition significantly

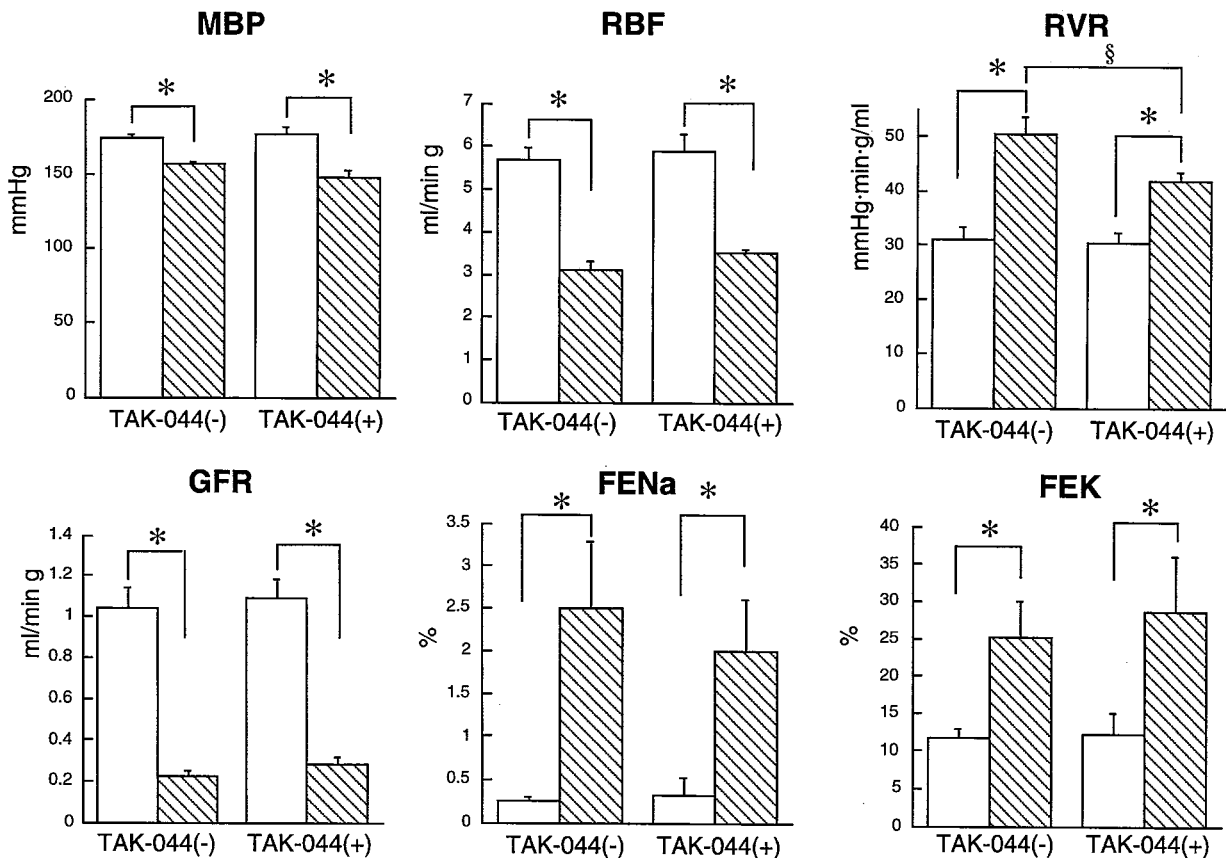


Fig. 1. Effects of TAK-044 on FK506-induced renal functional changes. MBP, mean blood pressure; RBF, renal blood flow; RVR, renal vascular resistance; GFR, glomerular filtration rate; FENa, fractional excretion of sodium; FEK, fractional excretion of potassium. □: vehicle, ▨: FK506. Values shown are means±S.E. *, §P<0.05, comparison between indicated groups.

decreased in FK506-treated groups (groups 3 and 4) compared with corresponding vehicle-treated groups (groups 1 and 2). Although TAK-044 did not affect MBP in FK506-treated rats, BP of TAK-044-treated rats without FK506 (group 2) was slightly lower than that of corresponding control rats (group 1). Plasma creatinine concentration and BUN significantly rose 7 days after the start of FK506 administration and further increased on the 14th day. Ccr was significantly decreased with FK506 treatment. Fractional excretion of Na (FENa) did not change at 7 days after FK506 treatment but increased on the 14th day. Similarly, fractional excretion of K (FEK) was higher in FK506-treated groups (groups 3 and 4). PRA was increased with FK506 (Table 3). However, these FK506-induced alterations were not significantly affected by the treatment with TAK-044. The 24-hr trough concentration of FK506 was 20.8 ± 1.2 ng/ml, which was not significantly affected by TAK-044 treatment (Table 3).

Renal hemodynamic study: Figure 1 shows renal hemodynamics and renal tubular function obtained from anesthetized SHR following daily administration of FK506 for 14 days. MBP was lower in FK506-treated rats compared to corresponding vehicle-treated rats. RBF was significantly lower in FK506-treated groups. Calculated RVR increased with FK506 treatment. GFR was markedly depressed when rats were treated with FK506. Although administration of TAK-044 did not affect RBF and GFR significantly in either the FK506 or vehicle treated group, TAK-044 significantly attenuated the FK506-induced increase in RVR (50.5 ± 3.1 vs 42.0 ± 1.4 mmHg·min·g/ml for group 3 vs group 4, $P < 0.05$). FENa and FEK were higher in FK506-treated groups but TAK-044 did not affect these changes. Hematocrit values

were the same among the groups; group 1, $51.7 \pm 1.5\%$ ($n=3$); group 2, $50.8 \pm 0.6\%$ ($n=4$); group 3, $51.0 \pm 0.4\%$ ($n=5$); group 4, $51.3 \pm 0.5\%$ ($n=4$).

Effectiveness of chronic ET receptor blockade: Baseline MBP, RBF and RVR were 163 ± 6 mmHg, 7.6 ± 0.3 ml/min and 21.5 ± 1.4 mmHg·min·g/ml in saline-treated rats and 166 ± 4 mmHg, 7.0 ± 0.8 ml/min and 24.8 ± 3.2 mmHg·min/ml in rats receiving TAK-044 (3 mg/day, 14 days). Intravenous administration of ET-1 elicited a transient decrease followed a sustained increase in BP. RBF was decreased with ET-1. Depressor and pressor responses and the increases in RVR following ET-1 challenge are summarized in Fig. 2. In rats treated with saline, depressor responses to ET-1 (0.1, 0.3 and 1.0 nmol/kg) were -62 ± 3 , -78 ± 6 and -65 ± 4 mmHg, respectively. In rats treated with TAK-044, depressor responses were significantly attenuated and were -12 ± 3 , -33 ± 2 and -43 ± 2 mmHg, respectively. Pressor responses to ET-1 (0.1, 0.3 and 1.0 nmol/kg) were dose-dependent and were 8 ± 2 , 15 ± 2 and 41 ± 3 mmHg in saline-treated rats, respectively; they were significantly attenuated to be 1 ± 1 , 3 ± 1 and 20 ± 5 mmHg by TAK-044 treatments. ET-1 at doses of 0.1, 0.3 and 1.0 nmol/kg dose-dependently increased RVR by 8 ± 0 , 21 ± 4 and 98 ± 18 mmHg·min·g/ml in saline treated rats, respectively. When rats were treated with TAK-044, corresponding values were 2 ± 1 , 9 ± 2 and 29 ± 9 mmHg·min·g/ml, significantly less than those in saline-treated rats.

Effect of FK506 on ET-1 mRNA expression

Northern blot analyses of mRNA for ET-1 and GAPDH in the whole kidney, renal cortex and medulla

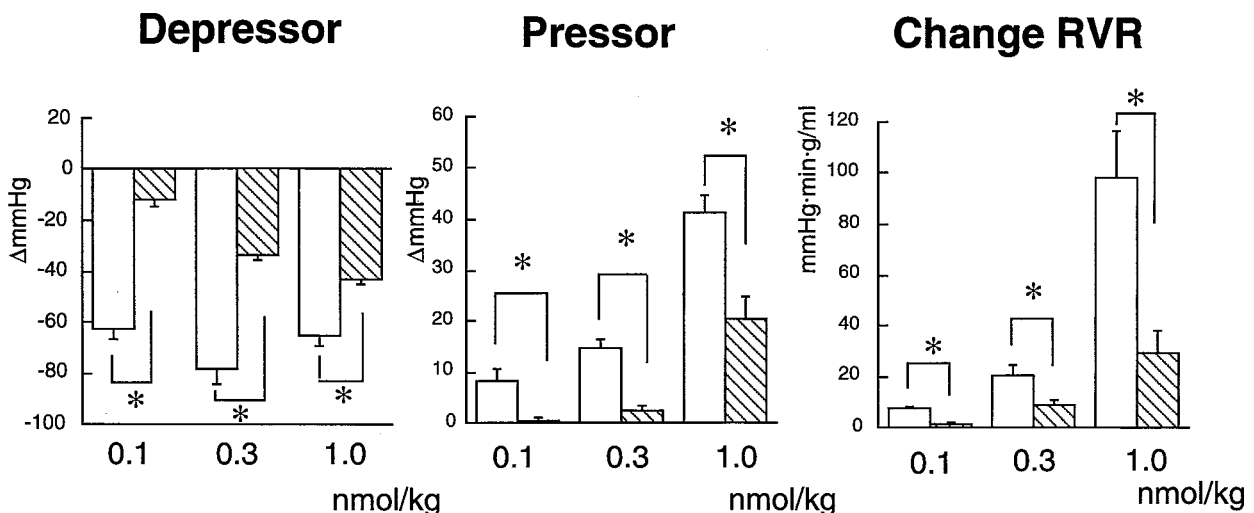


Fig. 2. Effects of TAK-044 on systemic and renal hemodynamic response to exogenous ET-1. RVR, renal vascular resistance. □: saline ($n=5$), ▨: TAK-044 ($n=4$). Values shown are means \pm S.E. * $P < 0.05$, comparison between indicated groups.

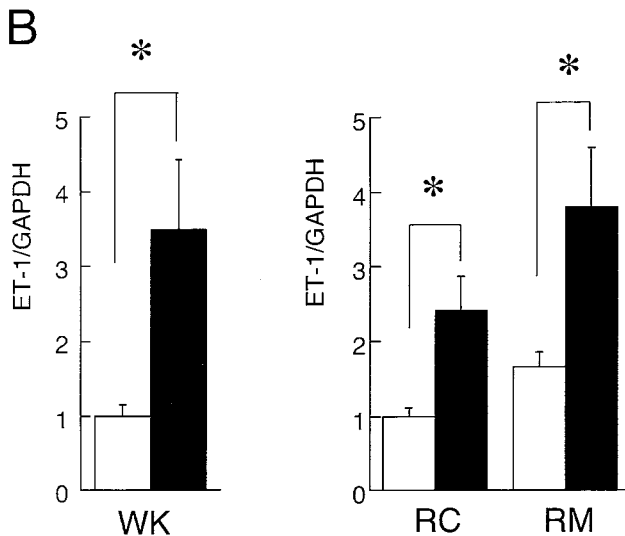
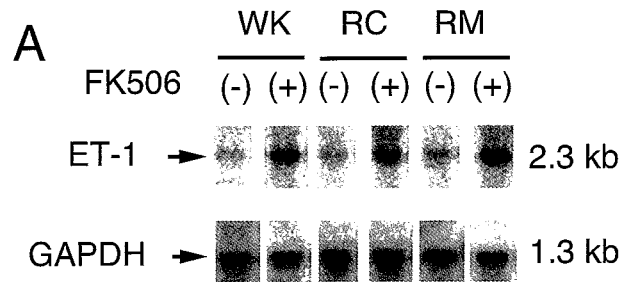


Fig. 3. Renal ET-1 mRNA levels following FK506 treatment. A: Autoradiograms of Northern blot analysis of mRNA of renal ET-1 and GAPDH following FK506 treatment. WK, whole kidney; RC, renal cortex; RM, renal medulla. B: Effects of FK506 on mRNA levels of ET-1 in the kidney. The ordinate shows each mRNA level, corrected for GAPDH mRNA level. □: vehicle, ■: FK506. Each bar represents the means \pm S.E. * $P < 0.05$, comparison between indicated groups.

from rats treated with vehicle or FK506 are shown in Fig. 3. Quantification by a bioimaging analyzer demonstrated a significant increase in ET-1 mRNA level in the whole kidney (3.3-fold) and in both the renal outer cortex (2.2-fold) and medulla (2.7-fold) in FK506-treated rats (group 3) compared to the vehicle-treated rats (group 1). The mRNA level of ET_B receptor was not affected by FK506 treatment (Fig. 4). The signal of ET_A-receptor mRNA was so weak that we could not perform a quantitative analysis.

DISCUSSION

Although FK506, a potent immunosuppressant, often causes nephrotoxicity, its mechanism has been poorly understood partly due to the lack of good animal models. In the present experiment, we tested the role of ET in

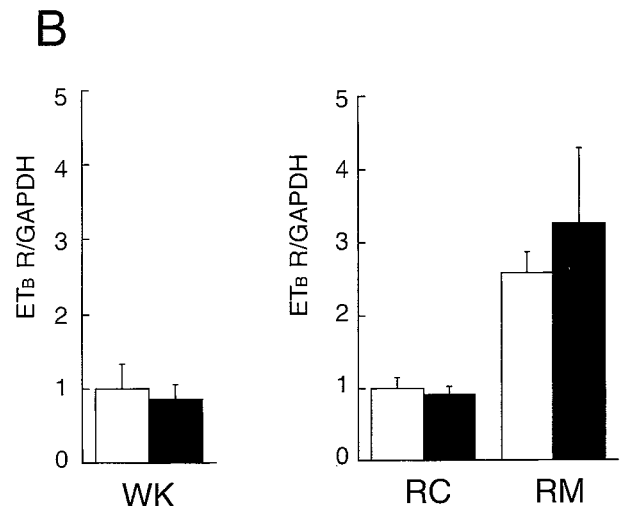
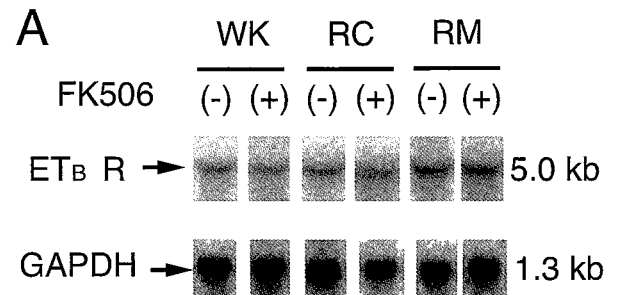


Fig. 4. Renal ET_B receptor mRNA levels following FK506 treatment. A: Autoradiograms of Northern blot analysis of mRNA of renal ET_B receptor and GAPDH following FK506 treatment. WK, whole kidney; RC, renal cortex; RM, renal medulla. B: Effects of FK506 on mRNA levels of ET_B receptor in the kidney. The ordinate shows each mRNA level, corrected for GAPDH mRNA level. □: vehicle, ■: FK506. Each bar represents the means \pm S.E.

FK506-induced renal dysfunction by using SHR. We demonstrate here that ET is responsible at least in part for FK506-induced renal hemodynamic deterioration but not tubular dysfunction.

Daily administration of FK506 to SHR for 2 weeks produced progressive renal functional deteriorations as reflected by the increase in plasma creatinine concentration and a decrease in Ccr without any appreciable enzymuria, confirming the results by Mitamura et al. (27). Though the dose of FK506 we used (4 mg/kg/day) appears to be much higher than clinical dose and may be criticized to be a pharmacological dose, the 24-hr trough concentration in the whole blood was only slightly higher than the therapeutic range (5–20 μ g/l) (36). Our present results using anesthetized rats clearly show that this in vivo manifestation of FK506-induced nephrotoxicity is characterized by elevated RVR and reduced GFR. As

increases in FENa and FEK were observed with FK506 treatment in both conscious and anesthetized conditions, tubular dysfunction is associated with renal hemodynamic deterioration. Since FK506 was administered intramuscularly, one may criticize that FK506 damaged the skeletal muscle at the site of injection, leading to kidney failure. However, this possibility is unlikely since histological findings by Mitamura et al. (27) who used the same method of administration in SHR did not show any sign of myoglobinuric nephropathy. Furthermore, plasma Na and K concentrations and hematocrit values were unchanged with FK506, supporting that the nephrotoxicity seen in the present experiment was elicited by FK506 itself but not by muscular damage or dehydration.

We found that ET-1 mRNA in the kidney increased following daily FK506 administration for 2 weeks. This is the first demonstration that ET-1 gene expression is increased during *in vivo* manifestation of FK506-induced nephrotoxicity. Although the plasma concentration of ET-1 was not increased, renal ET-1 content tended to increase ($P=0.078$). The failure to detect a significantly elevated ET-1 peptide level may be because endothelial cells mainly release ET-1 abluminally and that renal contents include not only *de novo* synthesized ET-1 but also the receptor-bound one. Alternatively, the fact that kidney actively degrades ET-1 by enzymes such as neutral endopeptidase (37) may explain the failure to detect any significant increase in renal ET-1 content despite the increase in renal ET-1 mRNA level.

Endothelin is a potent vasoconstrictor, and the renal vasculature is highly sensitive to ET (38). Although previous reports including ours mentioned that ET elicits renal vasoconstriction via ET_A receptors and renal vasodilation through activation of ET_B receptors (39–41), recent studies have shown that ET_B receptors are also involved in renal vasoconstriction (30, 42). In fact, the renal vasoconstrictor action of ET-1 was not blocked by BQ-123, an ET_A -receptor antagonist, but was markedly attenuated with the combination of BQ-123 and PD142893, an ET_A/ET_B -non-selective antagonist (11). Therefore, to elucidate the role of ET in FK506-induced renal dysfunction, TAK-044, an ET_A/ET_B -non-selective ET-receptor antagonist, was administered continuously by using an osmotic minipump at a rate of 3 mg/day for 2 weeks. With this maneuver, TAK-044 effectively blocked not only the systemic pressor and depressor action but also the renal vasoconstrictor action of exogenously administered ET-1. TAK-044 had little influence on basal BP (approximately 10 mmHg decrease) or renal hemodynamics of SHR. Thus our present results suggest a minimal role of ET in regulating systemic and renal hemodynamics in SHR, while previous reports were controversial (43–46). Studies with anesthetized SHR in the

present experiments reveal that FK506-induced increase in RVR was significantly attenuated by TAK-044. This attenuation does not appear to be due to the effect of TAK-044 on the pharmacokinetics of FK506 since the 24-hr trough concentration was not affected with TAK-044. Blockade of the renin-angiotensin system by TAK-044 is also unlikely since increased PRA by FK506 treatment was not affected with TAK-044. Therefore, ET is involved, at least in part, in the renal vasoconstriction induced by FK506 treatment. In contrast, GFR was decreased with FK506 treatment and TAK-044 did not prevent it, which is consistent with the results observed in the metabolic cage study. The reason why TAK-044 partially attenuated FK506-induced renal vasoconstriction but did not decrease GFR is difficult to explain. However, we previously reported that intrarenal arterial infusion of a small amount of ET-1 in the dog (38) elicited a decrease in RBF without any changes in GFR, which may explain the result. In addition other factors such as sympathetic nerve activation, increased thromboxane A_2 or activation of renin-angiotensin system may be involved in the FK506-induced renal hemodynamic deterioration, as was proposed previously to play a role in CsA-induced nephrotoxicity (47–49). FK506-induced increases in FENa and FEK were not affected with TAK-044, suggesting that ET is not involved in the FK506-induced tubular dysfunction.

Concomitantly with the renal dysfunction, systemic BP was decreased by 20 mmHg following administration of FK506 for 2 weeks. This reduction in BP was not caused by anesthesia since BP obtained from conscious rats shows similar effects of FK506 treatment. This is in marked contrast with the finding that chronic treatment of SHR with CsA accelerates BP elevation (50). CsA elicited moderate hypertension in rats that was ameliorated by ET-receptor antagonists (16, 17). These reports together with our present results may suggest the different contribution of ET to BP response following CsA and FK506 treatments. This notion was supported by our finding that TAK-044 did not affect BP following FK506 treatment. Although the reason for BP reduction by FK506 remains to be established, a clinical study indicates that hypertension is less frequent with FK506 than with CsA (3). At least, FK506-induced suppression of sympathetic outflow appears unlikely as heart rate was unchanged with FK506 treatment.

In the present experiments, we found that ET-1 mRNA was increased by FK506 not only in the renal medulla but also in the cortex. This is somewhat different from the previous reports observed with CsA treatment (15, 51) Iwasaki et al. (51) found an increase in ET-1 mRNA in the renal medulla but not in the glomeruli following single intravenous injection of CsA. Abassi et al. (15) found

that daily CsA treatment for 6 days elicited an increase in ET-1 mRNA in the medulla but not in the cortex. Although the site of enhanced gene expression of ET-1 within the cortex remains to be established, a unique mechanism of action of FK506 toward renal cortical tissue may be operating (52). In contrast to ET-1 gene expression, mRNA of the ET_B receptor was not affected by FK506 treatment within the kidney. It was reported that CsA up-regulates ET_B receptors in the medulla (53). We do not have any explanation for the discrepancy but the difference in drugs may be one of the reasons. Acute bolus injection of CsA to either SD rats or Wistar rats and acute exposure of isolated renal microvessels to CsA elicits ET-dependent renal vasoconstriction (4, 5, 7–10), whereas FK506 had no acute effect on renal hemodynamics even when 5 mg/kg (present experiments) or 20 mg/kg (25, 29) was acutely administered intravenously. These results suggest different regulation of ET production exists in CsA and FK506-induced nephrotoxicity. There are several studies on the effects of FK506 on ET release (21–25), but conflicting data were obtained. ET-1 release was increased (21) or unaffected (24) from a renal tubular cell line, LLC-PK1, by FK506. FK506 stimulated (23) or had no effects on ET release (25) from cultured endothelial cells, although another potent immunosuppressant, CsA consistently stimulates ET-1 release from cultured cells (18–20, 24, 25, 53).

The immunosuppressive mechanism of FK506 and CsA is primarily similar through inhibition of phosphatase calcineurin in the T lymphocyte (1, 2) and renal functional and histological disorders are considered basically the same (54–56). However, several lines of evidence show some differences in mechanism of nephrotoxicity between FK506 and CsA. For instance, CsA but not FK506 dose-dependently inhibits IL-1 β -dependent inducible nitric oxide synthase in renal mesangial cells (57). In the mouse model of FK506-induced nephrotoxicity, glomerular lesion was observed that was not seen with CsA (52). These reports and the present experimental results may suggest a possible mechanism(s) of nephrotoxicity unique to FK506 that remains to be established.

In summary, although ET is involved, in part, in the FK506-induced renal hemodynamic disorder, ET does not appear to be a major factor in eliciting FK506-induced nephrotoxicity.

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