Mechanisms of FK 506–Induced Hypertension in the Rat

Yoshiyu Takeda, Isamu Miyamori, Kenji Furukawa, Satoru Inaba, Hiroshi Mabuchi

Abstract—Tacrolimus (FK 506) is a powerful, widely used immunosuppressant. The clinical utility of FK 506 is complicated by substantial hypertension and nephrotoxicity. To clarify the mechanisms of FK 506–induced hypertension, we studied the chronic effects of FK 506 on the synthesis of endothelin-1 (ET-1), the expression of mRNA of ET-1 and endothelin-converting enzyme-1 (ECE-1), the endothelial nitric oxide synthase (eNOS) activity, and the expression of mRNA of eNOS and C-type natriuretic peptide (CNP) in rat blood vessels. In addition, the effect of the specific endothelin type A receptor antagonist FR 139317 on FK 506–induced hypertension in rats was studied. FK 506, 5 mg · kg⁻¹ · d⁻¹ given for 4 weeks, elevated blood pressure from 102±13 to 152±15 mm Hg and increased the synthesis of ET-1 and the levels of ET-1 mRNA in the mesenteric artery (240% and 230%, respectively). Little change was observed in the expression of ECE-1 mRNA and CNP mRNA. FK 506 decreased eNOS activity and the levels of eNOS mRNA in the aorta (48% and 55%, respectively). The administration of FR 139317 (10 mg · kg⁻¹ · d⁻¹) prevented FK 506–induced hypertension in rats. These results indicate that FK 506 may increase blood pressure not only by increasing ET-1 production but also by decreasing NO synthesis in the vasculature. (Hypertension. 1999;33:130-136.)

Key Words: FK 506  endothelin receptor antagonist  endothelin  nitric oxide  hypertension  endothelin-converting enzyme  natriuretic peptides

T acrolimus (FK 506), a macrolide compound isolated from Streptomyces tsukubaensis, has potent immunosuppressive properties. FK 506 is 10 to 100 times more potent than cyclosporin (CysA). The nephrotoxic side effects of CysA and the development of hypertension observed during CysA treatment also seem to be characteristic side effects of FK 506. The incidence of hypertension in previously normotensive liver or heart transplant recipients treated with CysA has been reported to be 80% to 90%. A lower incidence (50% to 70%) of hypertension in FK 506–treated recipients has been reported. Although renal dysfunction is usually also present in transplant recipients treated with CysA or FK 506, blood pressure elevations have been observed in the absence of detectable renal dysfunction. The exact or FK 506, blood pressure elevations have been observed in the absence of detectable renal dysfunction. The exact mechanism of FK 506–induced hypertension is unclear, but several researchers have suggested that the primary pathogenic mechanism may be vascular. We have reported that CysA and FK 506 both increased the synthesis of endothelin-1 (ET-1) in cultured vascular endothelial cells.

The natriuretic peptides organize a family of 3 distinct peptides (atrial natriuretic peptide [ANP], brain natriuretic peptide [BNP], and C-type natriuretic peptide [CNP]) and are involved in body fluid homeostasis and blood pressure control. CNP is reported to be synthesized in the vascular endothelial cells and to possess local effects of vascular tone and remodeling. Nitric oxide (NO) is synthesized from L-arginine by 2 enzymes. The generation of NO by constitutive, Ca²⁺-dependent NO synthase (NOS) from the vascular endothelium plays an important role in the homeostasis of the vascular system. Three isoforms of NOS have been cloned from rat brain (nNOS), vascular endothelium (eNOS), and inducible Ca²⁺-independent enzyme (iNOS). Vascular eNOS maintains vascular tone by releasing small amounts of NO in response to receptor stimulation and shear stress. This is clearly illustrated by the fact that the inhibition of NOS leads to generalized vasoconstriction and a significant hypertensive response. To clarify the mechanisms of FK 506–induced hypertension, we studied the chronic effects of FK 506 on the synthesis of ET-1, the expression of messenger RNA (mRNA) of ET-1 and endothelin-converting enzyme-1 (ECE-1), the eNOS activity, and the expression of mRNA of eNOS and CNP in rat blood vessels. In addition, the effect of the specific endothelin type A (ETₐ) receptor antagonist FR 139317 on FK 506–induced hypertension in rats was studied.

Methods

Animals and Experimental Protocol

All experiments were performed according to guidelines for the use of experimental animals of the Animal Research Committee of Kanazawa University. Male Wistar-Kyoto rats (weighing 200 to 220 g) were housed in metabolic cages with free access to tap water and normal rat chow (0.1 mmol/g Na and 0.24 mmol/g K; Nippon Charles River, Kanagawa, Japan). The rats were divided into 5 experimental groups. Group 1 received daily oral administration of FK 506 (Fujisawa, Osaka, Japan) solubilized in 0.5% Arabic gum

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solution at a dose of 5 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \) for 4 weeks (n=60); group 2 received daily oral administration of FK 506 at a dose of 0.5 mg/kg for 4 weeks (n=60); group 3 (n=60) was given FR 139317, an ET\(_x\) receptor antagonist, kindly donated by Fujisawa Pharmaceutical Co, Osaka, Japan,\(^{15} \) injected subcutaneously at a dose of 10 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \) for 4 weeks. Group 4 (n=60) received a combination of FK 506 (5 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \)) with FR 139317 (10 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \)) for 4 weeks. Control rats (n=60) received the vehicle alone for 4 weeks.

The blood pressure was determined by the plethysmographic tail-cuff method as previously reported.\(^{14} \) Blood was collected from the tail vein as previously reported.\(^{14} \) Plasma ET-1 concentrations were determined using a sandwich-type enzyme immunoassay after extraction with a Sep-Pak C18 cartridge column (Waters Associates, Milford, MA).\(^{15} \) Serum creatinine concentrations were determined by the alkaline picrate method. The FR 139317 plasma concentration was measured using a high-performance liquid chromatography system as previously reported.\(^{15} \)

**Experiments of Perfusion of the Rat Mesenteric Artery**

Eight rats from each group were used for experiments involving mesenteric arterial perfusion. After the rat was put under pentobarbitonal anesthesia, the superior mesenteric artery was isolated at its junction with the abdominal aorta and freed of fat and connective tissue by the method of McGregor,\(^{16} \) with minor modifications as we have previously reported.\(^{17} \) Briefly, the isolated artery with the second branch was perfused with Krebs-Ringer solution, pH 7.4, at a temperature of 37\(^{\circ}\)C and oxygenated with a 95\% O\(_2\)-5\% CO\(_2\) gas mixture at a constant flow rate of 4 mL/min. Perfusion pressure was constantly monitored and recorded by means of a pressure transducer connected to a polygraph (model RM 600; Nihon-Koden, Tokyo, Japan).

**Measurements of ET-1 in the Perfusate**

After 30 minutes of equilibration, the perfusate was collected for 1 hour. The perfusate was extracted with a Sep-Pak C18 cartridge in preparation for chromatography using a reverse-phase high-performance liquid chromatographic system. Measurements of ET-1 in the perfusate were performed as previously reported.\(^{18} \) After these experiments on the perfusate, the mesenteric artery was homogenized in 10 mL Krebs-Ringer buffer solution in a tissue grinder. Protein assays were performed according to the method of Bradford.\(^{19} \)

**Quantification of eNOS Activity**

Fifteen rats from each experimental group were used for the quantification of eNOS activity in aortic endothelial cells. After the rat was put under pentobarbitonal anesthesia, the aorta was excised and washed briefly in Krebs-Ringer solution gassed with 95\% O\(_2\)-5\% CO\(_2\). The NOS activity in aortic endothelial cells was measured as previously reported.\(^{20} \)

**Competitive Polymerase Chain Reaction for ET-1, ECE-1, and CNP mRNAs**

Seven rats from each group were used for the quantification of mRNA of ET-1, ECE-1, and CNP in the mesenteric arteries and for eNOS mRNA in the aortae. After the rat was put under pentobarbitonal anesthesia, the mesenteric arteries, including the first branch and the aortae, were removed immediately after the rats had been euthanized by decapitation and freed of fat and connective tissue. The tissue was promptly weighed, frozen in liquid nitrogen, and stored at −80°C prior to use. Total RNA from rat mesenteric arteries and aortae were separated with guanidinium thiocyanate followed by centrifugation in a cesium chloride solution. Quantification of ET-1, ECE-1, and CNP mRNAs was performed using the competitive polymerase chain reaction (PCR) method as previously reported.\(^{15} \) The sequences of sense and antisense primers for ET-1,\(^{21} \) ECE-1,\(^{22} \) and eNOS\(^{23} \) were designed as previously reported. The intra- and interassay variabilities of the competitive PCR were 11.9% and 12.9%, respectively, for ET-1, 12.2% and 13.4% for CNP, 12.0% and 13.1% for ECE-1, and 12.3% and 13.9% for eNOS.

**Southern Blot Analysis**

The RT-PCR products in 10-μL aliquots were electrophoresed on a 3% agarose gel and transferred to nylon membranes. Hybridization was performed as previously reported\(^{26} \) with the specific oligoprobes for ET-1 (5’-CAAAGAAGACTCCGAGCCCAAA-3’), CNP (5’-AAGGAGAGGCCATCGGTCTTGCT-3’), and ECE-1 (5’-GGCTACCCCAACTTCATCAT-3’) that had been end-labeled with [\(^{32}\)P]-dCTP (6000 Ci/mmol, New England Nuclear) using a 5’-end oligonucleotide labeling kit.

Data are expressed as mean±SEM. The significance of differences was assessed by 1-way ANOVA and multiple comparisons procedures. Statistical significance was accepted at a level of \( P<0.05 \).

**Results**

**Chronic Effects of FK 506 on Blood Pressure**

Chronic administration of FK 506 (5 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \)) significantly increased blood pressure beginning at 2 weeks compared with control rats or rats treated with 0.5 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \) of FK 506 (Figure 1). Treatment with FR 139317 resulted in a 60% and 70% decrease in the FK 506–control difference in blood pressure at 3 and 4 weeks, respectively (Figure 1).

**Chronic Effects of FK 506 on Renal Function and ET-1 Levels in Plasma and Perfusate**

Table 1 summarizes body weight, heart rate, serum creatinine concentration, and ET-1 concentration in plasma and in the perfusate of the mesenteric artery in each experimental group. No significant differences in body weight or serum creatinine concentration between the experimental groups were observed. Plasma ET-1 concentrations were significantly higher in FK 506 (5 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \))-treated rats or in FR 139317–treated rats compared with control rats at 2 and 4 weeks (\( P<0.05 \)). ET-1 concentrations in the perfusate of the
The present study demonstrates not only that FK 506 increases blood pressure but also demonstrates that this blood pressure increase is due to an increase in endothelin production and a decrease in vascular synthesis of NO. The dose of 5 mg kg⁻¹ · d⁻¹ of FK 506, which we used, is reported to be sufficient to prevent rejection of allograft in rats. Compared with the clinical dose of FK 506 (0.2 to 0.3 mg kg⁻¹ · d⁻¹), this dose seems to be very high.

**Chronic Effects of FK 506 on the Expression of ET-1 and eNOS mRNAs**

The concentrations of ET-1 mRNA in the mesenteric arteries of FK 506–induced hypertensive rats were significantly increased as compared with control rats at 2 and 4 weeks (P<0.05; Figure 2). The levels of eNOS mRNA in the aortae of FK 506–induced hypertensive rats were significantly decreased as compared with control rats at 2 and 4 weeks (P<0.05; Figure 2).

**Chronic Effects of FK 506 on the Expression of CNP and ECE-1 mRNAs**

Table 2 summarizes the quantification of CNP and ECE-1 mRNAs in the mesenteric arteries of each experimental rat group. Treatment with FK 506 did not affect vascular CNP and ECE-1 mRNA levels.

**Discussion**

The mRNAs of CNP and ECE-1 were not affected by FK 506, indicating that the increase in ET-1 and decrease in eNOS observed in FK 506-treated rats were due to a decrease in the synthesis of NO

**Competitive PCR**

Figure 3 illustrates that increasing concentrations of each competitive template for ET-1, ECE-1, CNP, and eNOS from 0 to 160 × 10⁻³ progressively inhibited the amplification of endogenous cDNA of ET-1, ECE-1, CNP, and eNOS in blood vessels. When PCR was carried out in the absence of reverse transcription, bands were not seen at 471, 682, 597, or 214 bp.
reported that the effective dose of FK 506 in rats is more than 50 times that in human. The hypertensinogenic dose of CysA (20 to 25 mg · kg⁻¹ · d⁻¹) in rat is also higher than the clinical dose (2 to 3 mg · kg⁻¹ · d⁻¹) in humans.⁵,¹⁸ The dose of 0.5 mg · kg⁻¹ · d⁻¹ of FK 506 did not increase blood pressure. Ohara et al²⁰ reported the toxicological evaluation of FK 506, in which rats given 0.32 mg · kg⁻¹ · d⁻¹ of FK 506 orally did not show any toxicological effects.

The nephrotoxic side effect of FK 506 is common in transplant recipients.⁶ In our results, serum creatinine concentration did not increase in FK 506-treated rats; however, the possibility of renal injury by FK 506 was not ignored. Cvetkovic et al¹⁰ reported that rats that received FK 506 orally at a dose of 5 mg · kg⁻¹ · d⁻¹ for 4 weeks did not show any increase in the BUN and serum creatinine levels.

ET is a potent vasoconstrictor produced by endothelial cells. Its putative role in the pathogenesis of hypertension is supported by several lines of evidence suggesting that endothelial damage is generally associated with the enhanced release of this vasoconstrictor peptide.³¹ We have reported that ET-1 synthesis is increased in prehypertensively hypertensive rats (SHR)³² and cyclosporine-induced hypertensive rats.¹⁵,¹⁷,¹⁸ Recently, Niranjani et al³³ reported that endogenous expression of preproET-1 in rats, accompanied by an elevation of plasma ET-1 concentrations to the levels seen in pathophysiological states, can cause systemic hypertension through the activation of the ETα receptor. In the present study, ET-1 production from the vasculature and the levels of ET-1 mRNA in the vasculature of FK 506-induced hypertensive rats were increased compared with control rats. We have reported that FK 506 increased the production of ET-1 and the levels of ET-1 mRNA in human vascular endothelial cells.¹⁰ Moutabarrak et al¹⁴ also reported that FK 506 increased the secretion of ET-1 from cultured kidney cells and that oral administration of FK 506 elevated plasma ET-1 concentrations. The synthesis of bioactive ET-1 requires a recently cloned phosphoramidon-sensitive ECE.²⁴ ECE-1 converts an intermediate metabolite, big ET-1, into bioactive big ET-1. Recently, the ECE-1 gene in the kidney has been reported to be involved in the pathogenesis of hypertension in spontaneously hypertensive rats.¹⁵ The present findings that FK 506 increased the expression of ET-1 mRNA but not ECE-1 mRNA in the vasculature indicates that the production of ET by FK 506 is regulated at the transcription level of ET-1 mRNA rather than ECE-1. Abassi et al³⁶ also reported that the production of ET by CysA is regulated through the modulation of mRNA levels and not by regulation of ECE levels. In the present model we found that blockade of the ETα receptor by FR 139317 decreased the hypertensive effect of FK 506, indicating that FK 506 exerts its systemic pressor effect partly through activation of the ETα receptor.

In this study, FK 506 did not increase the expression of CNP mRNA in the vasculature, which may suggest local vascular CNP production possesses an insignificant role for FK 506-induced hypertension.

Alterations in the vascular endothelium (in particular, a deficiency in the L-arginine–NO pathway) have been suggested to play a major role in hypertension.³⁷ Vascular eNOS maintains vasodilator tone by releasing small amounts of NO in response to receptor stimulation and shear stress. This is clearly illustrated by the fact that inhibition of NO synthase leads to generalized vasoconstriction and a significant hypertensive response.³⁸ Studies in humans suggest that hypertension is associated with a decrease in NO generation.³⁹ In SHR, impaired NO synthesis has been reported by Koller and Huang.⁴⁰ Rees et al also have reported decreased eNOS activity in the aortae of hypertensive rats.⁴¹ In this study, Ca²⁺-dependent eNOS activity and mRNA levels were decreased in the aortae of FK 506-induced hypertensive rats. Thus, FK 506 may decrease NO production by inhibiting
eNOS activity in the vasculature and influence blood pressure. However, Stroes et al.\(^40\) reported that expression of eNOS mRNA in human umbilical endothelial cells is increased by CysA and proposed a protective effect of eNOS against CysA-associated vasoconstriction. Recent reports suggest potential roles of inducible NOS (iNOS) in the vasculature, including endothelial and smooth muscle cells.\(^37\) In our study, Ca\(^{2+}\)-independent eNOS activity was not affected by FK 506. Marumo et al.\(^41\) have reported that CysA inhibits iNOS in vascular smooth muscle cells and that FK 506 has no inhibitory effect on iNOS. However, an inhibitory effect of FK 506 on iNOS activity of cultured macrophages has been reported by Conde et al.\(^52\) Vascular gene transfer may serve as a tool with which to study vascular biology and may have therapeutic potential. A potential candidate for therapeutic vascular gene transfer is the enzyme eNOS. Kullo et al.\(^43\) have reported that overexpression of the eNOS gene in the endothelium of carotid arteries resulted in diminished contractile responses and enhanced endothelium-dependent relaxation. Further study of the effect of eNOS gene overexpression is needed to clarify the role of eNOS in FK 506–induced hypertension.

Scherrer et al.\(^44\) reported that heart-transplant recipients receiving CysA had higher blood pressures than those receiving azathioprine and prednisone. The incidence of hypertension in transplant recipients treated with FK 506 is lower than those treated with CysA. Canzanello et al.\(^45\) reported no significant differences of renal sodium handling with CysA and FK 506 after orthotopic liver transplantation. Although FK 506 and CysA are not structurally related and have different binding proteins mediating intracellular binding, most immunologic and intracellular actions appear to be closely parallel. Some authors have suggested that alterations of intracellular calcium localization may mediate the immunosuppressive and vascular effects of both agents.\(^46\) In this study, the mechanisms of FK 506–induced hypertension in the rat did not differ from reported mechanisms of CysA. Recently, calcineurin phosphatase inhibition by CysA or FK 506 has been postulated to lead to hypertension through the induction of transforming growth factor-beta and resultant arterial vasoconstriction.\(^47\) However, Akita et al.\(^48\) have demonstrated the weaker effect of FK 506 on NO production in vascular smooth muscle cells as compared with CysA. We and others have reported that a therapeutic dose of FK 506 increased secretion of ET-1 less than a therapeutic dose of CysA from vascular endothelial cells\(^10\) or cultured kidney cells.\(^34\) These findings suggest that the lower incidence of complications seen in FK 506 is due in part to the use of a lower clinical dose compared with that of CysA.

Figure 3. Effect of concentration of the competitive template on the amplification of PCR products derived from ET-1, ECE-1, and CNP cDNAs of mesenteric arteries or aortic eNOS cDNA and each competitive template (Mimic).
TABLE 2. Concentration of ECE-1 mRNA and CNP mRNA in Mesenteric Arteries of Experimental Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>ECE-1 mRNA/100 ng RNA</th>
<th>CNP mRNA/100 ng RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.9 ± 0.4</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>FK 506 0.5</td>
<td>2.5 ± 0.3</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>FK 506 5</td>
<td>3.0 ± 0.5</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>FK 506 FR</td>
<td>2.4 ± 0.3</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>FK 506 + FR</td>
<td>2.5 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>4 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.7 ± 0.4</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>FK 506 0.5</td>
<td>2.9 ± 0.4</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>FK 506 5</td>
<td>3.3 ± 0.5</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>FK 506 FR</td>
<td>2.6 ± 0.4</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>FK 506 + FR</td>
<td>2.6 ± 0.3</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

Control indicates rats treated with vehicle; FR, rats treated with FR 139317; FK 506 + FR, rats treated with FK 506 (5 mg) plus FR 139317. Values are mean ± SEM; n = 7 for each group.

Recently, Krum et al demonstrated that long-term treatment with an ET receptor antagonist, bosentan, lowered blood pressure in patients with essential hypertension. We have reported that the ETα receptor antagonist FK 139317 prevents CysA-induced hypertension in rats. Benigni et al reported that a specific ETα receptor antagonist protects against the progression of CysA-induced renal dysfunction. Further study is necessary to understand the clinical implications of using an ETα receptor antagonist to prevent the complications of FK 506.

In conclusion, FK 506 may increase blood pressure not only by increasing ET production but also by decreasing NO synthesis in the vasculature. The specific ETα receptor antagonist FR 139317 may be useful in preventing the hypertension induced by FK 506.

References


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