Tacrolimus Induces Glomerular Injury via Endothelial Dysfunction Caused by Reactive Oxygen Species and Inflammatory Change

Kengo Kidokoro  Minoru Satoh  Hajime Nagasu  Takeo Sakuta
Atsunori Kuwabara  Daisuke Yorimitsu  Yuko Nishi  Naruya Tomita
Tamaki Sasaki  Naoki Kashihara

Department of Nephrology and Hypertension, Kawasaki Medical School Kurashiki, Okayama, Japan

Key Words
Angiotensin II receptor blocker • Nephrotoxicity • NAD(P)H oxidase • Oxidative stress • Tacrolimus

Abstract

Background/Aims: The immunosuppressive drug tacrolimus (FK506) is used clinically to reduce the rejection rate in patients with kidney transplantation; however, the resultant nephrotoxicity remains a serious problem. In the present study we attempted to elucidate the mechanisms of glomerular injury induced by FK506 and the renoprotective effects of the angiotensin II receptor blocker telmisartan. Methods: Seven-week-old male Wistar rats were divided into three groups: vehicle group, FK506 group, and FK506 + telmisartan group. After 8 weeks, we assessed kidney function and renal morphological changes including oxidative stress. We also assessed the effect of FK506 in human glomerular endothelial cells (hGECs) with regard to reactive oxygen species (ROS). Results: FK506 induced ROS production via activation of NAD(P)H oxidase in the glomeruli. Expression of ICAM mRNA was increased in glomeruli from the FK506 group. These effects resulted in macrophage infiltration into the glomeruli. FK506 directly promoted NAD(P)H oxidase activity and accelerated production of ROS in hGECs. Conversely, cotreatment with telmisartan inhibited both NAD(P)H oxidase activity and production of ROS. Conclusion: These findings suggest that glomerular injury resulting from FK506 is caused by oxidative stress mediated by activation of NAD(P)H oxidase and that telmisartan exerts a renoprotective effect via antioxidative activity.

Introduction

The calcineurin inhibitor (CNI) tacrolimus (FK506) is used clinically to reduce the rejection rate in patients with kidney transplantation. Recently, CNIs have become pivotal for the prevention of allograft rejection [1]. FK506 is more potent than cyclosporine A in the suppression of T cell activation and is often used when cyclosporine A is not effective against acute rejection or when intolerance is an issue [2, 3]. However, the nephrotoxicity resulting from FK506 remains a serious problem.

Acute CNI nephrotoxicity is considered a functional impairment of renal hemodynamics caused by vasoconstriction of afferent arterioles in response to use of this class of drug [4]. Thus, the acute form of nephrotoxicity is reversible. However, long-term use of CNI is associated with irreversible renal dysfunction attributable to progressive forms of tubulointerstitial and glomerular inju-
ry. The histological changes typically observed in chronic CNI nephrotoxicity cases include arteriolar hyalinosis, tubular atrophy, interstitial fibrosis, thickening and fibrosis of the Bowman’s capsule, and focal or global glomerular sclerosis [5]. Chronic tacrolimus nephropathy is also associated with arteriolopathy and narrowing of the arteriolar lumen, which are major contributors to the development of interstitial fibrosis, tubular atrophy, and glomerular sclerosis [6].

Multiple factors have been reported to mediate the pathogenic mechanisms underlying CNI nephrotoxicity and include increased production of vasoconstriction factors, such as endothelin or thromboxane, and a decrease in vasodilation factors like prostacyclin, prostaglandin E2, and nitric oxide. Activation of the renin-angiotensin system (RAS) has also been reported to mediate CNI nephrotoxicity [7–9]. In addition, several reports indicate that CNIs increase superoxide production, likely through vasoconstriction-associated hypoxia and/or direct effects [10, 11].

We have shown that increased production of reactive oxygen species (ROS) leads not only to the alteration of renal hemodynamics, but also to the development and progression of glomerular injury in various disease models [12]. In addition, the RAS is implicated in the generation of ROS through activation of NAD(P)H oxidase. We also clarified that angiotensin receptor blockers (ARB) ameliorate glomerular injury by suppression of oxidative stress [13]. CNIs have been indicated to promote generation of ROS in a number of cell types including mesangial cells [14, 15]. Based on these findings, we hypothesize that FK506 enhances glomerular oxidative stress resulting in glomerular injury. We have attempted to elucidate the mechanisms of glomerulopathy induced by FK506 and the renoprotective effects of the ARB telmisartan.

Materials and Methods

Experimental Protocol and Tissue Preparation

The experimental protocol (No. 08-067) was approved by the Ethics Review Committee for Animal Experimentation of the Kansai Medical School, Kariyado, Japan. Male Wistar rats, purchased from Charles River Japan (Kanagawa, Japan), were housed in a temperature- and humidity-controlled room with a 12-hour light-dark cycle; they were fed standard laboratory animal chow and had free access to tap water. Seven-week-old Wistar rats (body weight 200–230 g) were randomly divided into the following three groups (n = 8 in each group): castor oil and ethanol (vehicle for FK506; control group), FK506 at 0.6 mg/kg/day (FK506 group), and FK506 plus telmisartan at 1 mg/kg/day (FK506 + Telm group). The dosage of 0.6 mg/kg/day FK506 was determined by preliminary experiments. This dosage does not induce diarrhea or hypertension in rats. The dosage of 1.0 mg/kg/day telmisartan was also determined by preliminary experiments. During the experimental period, body weight was measured weekly. Systolic arterial blood pressure was also measured weekly using the tail-cuff method at an ambient temperature of 37°C (BP-98A; Softron, Tokyo, Japan) [16]. The rats were subjected to a 24-hour fast in metabolic cages to collect urine samples on the day before they were killed. After 9 weeks of treatment, the rats were killed and blood samples were obtained using an 18-gauge needle inserted into the left ventricle. Urinary protein excretion and levels of serum and urinary creatinine were measured. The abdominal aorta was cannulated, and the kidney was perfused in a retrograde manner with ice-cold phosphate-buffered saline (PBS; pH 7.4). One-quarter of each removed kidney was immersed and fixed in 4% paraformaldehyde and then embedded in paraffin. The remainder of each kidney was cut into small pieces, and glomeruli were isolated by mechanical graded sieving [17] for superoxide production assay, NAD(P)H oxidase activity assay, and mRNA extraction.

Histological Examination

Sections (4-μm thick) were prepared from renal tissue samples embedded in paraffin and stained with periodic acid-Schiff (PAS) and Masson’s trichrome. Kidney sections were photographed and digitized into color images using a Nikon Coolscope (Nikon, Tokyo, Japan).

Detection of Superoxide in Glomeruli

Superoxide production was detected by 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, Ore., USA) for 10 min and then rinsed with PBS. Fluorescence images were obtained using a confocal laser microscope (TCS SP2 AOBS MP; Leica Microsystems, Tokyo, Japan) at excitation/emission wavelengths of 485/535 nm for DCF. The fluorescence intensity values from 20 different isolated glomeruli were calculated by Leica TCS-NT software (Leica Microsystems) and presented as average values.

Detection of ED-1-Positive Cells in Glomeruli

Macrophages were detected by ED-1 staining. Paraffin-embedded kidney sections approximately 4-μm thick were deparaffinized. A rat anti-ED-1 monoclonal antibody (Serotec, Oxford, UK) was used as the primary antibody. Antibody binding was detected using a Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan) and 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, Mo., USA). We examined 30 glomeruli of each rat (240 glomeruli per group) and counted ED-1-positive cells under a light microscope. The average number of ED-1-positive cells per glomerulus was compared.

Effect of NAD(P)H Oxidase Activity Induced by FK506

We used human glomerular endothelial cells (hGECs), passage 8–10 (Cell Systems Corp., Kirkland, Wash., USA). Cells were cultured on 10-cm dishes and propagated in Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated newborn bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in CO2. The hGECs were incubated with: castor oil and ethanol (vehicle for FK506); FK506 (10⁻⁷ M); FK506 (10⁻² M) + telmisartan (10⁻⁵ M or 10⁻⁴ M); or FK506 (10⁻² M) ± gp91tat (5 × 10⁻⁷ M)
10⁻⁵ M), a NAD(P)H oxidase inhibitor [19] for 24 h. In another in vitro experiment, hGECs were incubated with FK506 (10⁻⁷ M) and valsartan (10⁻⁶ or 10⁻⁵ M) or GW9662 (2 x 10⁻⁶ M), a PPAR-γ inhibitor. After incubation, NAD(P)H oxidase activity was measured using a lucigenin chemiluminescence assay.

Lucigenin Chemiluminescence Assay for Measuring NAD(P)H Oxidase Activity in Isolated Glomeruli and hGECs

NAD(P)H oxidase activity in the glomeruli and hGECs was measured in units per minute per milligram using lucigenin chemiluminescence as previously described [20].

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from the glomeruli and hGECs with TRizol (Invitrogen Japan, Tokyo, Japan). Reverse transcriptase reactions were performed using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare Bio-Sciences, Tokyo, Japan) for first-strand cDNA synthesis. Real-time quantitative PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). Primers and probes for TaqMan analysis were designed using Primer Express 1.5 (Applied Biosystems) with information from the supplier based on the sequence information from GenBank or EST databases. We have previously described the primers and probes used for gp91phox, p22phox, p47phox, p67phox (where phox indicates phagocyte oxidase), and angiotensin II type 1 receptor (AT1R) [12, 17]. Primer and probe sequences for rat ICAM-1 (NM_012967) were as follows: 5'-GAATCCAGCCCCTAATCTGA-3' (forward primer); 5'-TAAAGGCCAGGCATTTGTAG-3' (reverse primer); and 5'-FAM-CCCACTTGAAGTCTG-TAMRA-3' (TaqMan probe). Primer and probe sequences for rat VCAM-1 (NM_012889) were as follows: 5'-GGGAAACATGGAAGAGG-AATC-3' (forward primer); 5'-ATCCAGGACCACAACCTT-GAC-3' (reverse primer); and 5'-FAM-ATTACATTGCAGTGGCCTCAG-TAMRA-3' (TaqMan probe). Primer and probe sequences for human ICAM-1 (NM_000201) were as follows: 5'-CAAGGCCTCAGTCAGTG-TGA-3' (forward primer); 5'-CCTC-TGGCTTTGTCAAGATC-3' (reverse primer); and 5'-FAM-ATTACATTGCAGTGGCCTCAG-TAMRA-3' (TaqMan probe). Primer and probe sequences for human VCAM-1 (NM_001078) were as follows: 5'-ATTTG-GTACTGCTCTCATTG-3' (forward primer); 5'-CCTTCCCAT-TGATGGACTATC-3' (reverse primer); and 5'-FAM-CCCAA- TTTTTCTGTGAGAACC-TAM-RA-3' (TaqMan probe).

Statistical Analysis

Values are expressed as means ± standard error of the mean (SEM). Statistical comparisons were made using the Mann-Whitney U test or the one-factor analysis of variance with a Tukey-Kramer test for multiple comparisons. A p value of <0.05 was considered significant.

Results

Histological Examination

Histological analysis was performed on the renal cortex in tissue sections stained with PAS and Masson’s trichrome. There were no abnormalities in the glomeruli or tubulointerstitial areas in any groups (data not shown).

<table>
<thead>
<tr>
<th>Number</th>
<th>Control</th>
<th>FK506</th>
<th>FK506 + Telm</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>471 ± 37</td>
<td>396 ± 34*</td>
<td>397 ± 22*</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>131 ± 14</td>
<td>121 ± 8</td>
<td>123 ± 8</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>99 ± 10</td>
<td>98 ± 12</td>
<td>98 ± 7</td>
</tr>
<tr>
<td>S-CRN, mg/dl</td>
<td>0.29 ± 0.01</td>
<td>0.40 ± 0.08*</td>
<td>0.33 ± 0.06**</td>
</tr>
<tr>
<td>U-PROT/U-CRN, mg/g CRN</td>
<td>0.57 ± 0.07</td>
<td>0.42 ± 0.11</td>
<td>0.23 ± 0.05**</td>
</tr>
</tbody>
</table>

BW = Body weight; SBP = systolic blood pressure; DBP = diastolic blood pressure; S-CRN = serum creatinine; U-PROT = urinary protein excretion; U-CRN = urinary creatinine excretion. * p < 0.05 vs. Control; ** p < 0.05 vs. FK506.

Physiologic and Biochemical Parameters

Table 1 summarizes the physiologic and biochemical parameters in each group. Body weight was significantly decreased in the FK506 and FK506 + Telm groups compared with the control group (p < 0.05). Systolic blood pressure did not differ between groups. Serum creatinine was significantly higher in the FK506 group compared with the other two groups (p < 0.05). There was no difference in urinary protein excretion between the control and FK506 + Telm groups, but this parameter was significantly decreased in the FK506 + Telm group compared with the FK506 group (p < 0.05).

Glomerular ROS Production

Oxidation of DCFH to the fluorescent compound DCF was used as a qualitative marker of cellular oxidative stress, because a number of chemicals, such as H₂O₂, ONOO⁻, and HOCl, can produce DCF fluorescence [21]. Figure 1a shows representative glomerular fluorescence images in each group. DCF fluorescence intensity in the isolated glomeruli was significantly stronger in the FK506 group than in the control group, and weaker in the FK506 + Telm group than in the FK506 group. We determined NAD(P)H oxidase activity in isolated glomeruli with a lucigenin chemiluminescence assay (fig. 1b). NAD(P)H oxidase activity was also significantly higher in the FK506 group compared with the control group. Increased NAD(P)H oxidase activity induced by FK506 was partially suppressed by telmisartan treatment.

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tosolic components. Expression of mRNA of both cytosolic components was significantly increased in isolated glomeruli from the FK506 group. In contrast, expression of mRNA of both membrane components did not significantly differ between the groups (fig. 2a–d).

**Glomerular mRNA Expression of Inflammatory Markers**

Expression of ICAM-1 and VCAM-1 mRNA (fig. 3a, b) was increased in the FK506 group compared with the control group. Addition of telmisartan decreased mRNA expression of ICAM-1 and VCAM-1, and ICAM-1 expression was significantly decreased in the FK506 + Telm group compared with the FK506 group.

**FK506-Stimulated NAD(P)H Oxidase Activity in hGECs in vitro**

Using hGECs, we investigated the direct effects of FK506 on NAD(P)H oxidase activity in endothelial cells. NAD(P)H oxidase activity in hGECs was significantly increased in the FK506 group, and addition of telmisartan reduced FK506-stimulated NAD(P)H oxidase activity (fig. 6a). Gp91ptt almost completely inhibited FK506-
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Fig. 3. Expression of inflammatory markers in the glomerulus. The mRNA levels of ICAM-1 (a) and VCAM-1 (b) were quantitatively analyzed by real-time PCR. * p < 0.05 vs. control; † p < 0.05 vs. FK506.

Fig. 4. Macrophage infiltration in the glomerulus. a Immunohistochemical staining of ED-1 (marker for resident macrophages; brown color). Arrows show ED-1-positive cells. b Number of ED-1-positive cells per glomerulus. * p < 0.05 vs. control; † p < 0.05 vs. FK506.

Fig. 5. Angiotensin II type 1 receptor expression in the glomerulus. The mRNA level of angiotensin II type 1 receptor was quantitatively analyzed by real-time PCR. * p < 0.05 vs. control; † p < 0.05 vs. FK506.

stimulated NAD(P)H oxidase activity (fig. 6a), while in contrast to telmisartan, valsartan did not decrease NAD(P)H oxidase activity (fig. 6b). Furthermore, GW9662 abrogated the effects of telmisartan which inhibited NAD(P)H oxidase activity (fig. 6c).

FK506-Stimulated ICAM-1 mRNA Expression in hGECs in vitro

We also investigated the direct effect of FK506 on the expression of ICAM-1 and VCAM-1 mRNA in endothelial cells. ICAM-1 mRNA expression was increased in the FK506 group, and the addition of telmisartan reduced FK506-stimulated ICAM-1 mRNA expression (fig. 6d). The VCAM-1 mRNA expression level in hGECs was too low to detect (data not shown).
The aim of this study was to determine whether FK506 induced glomerular injury by enhancing glomerular oxidative stress. Our findings indicate that FK506 directly induces renal dysfunction and causes glomerular injury via ROS produced by NAD(P)H oxidase. Furthermore, the results of the present study suggest that FK506 induces glomerular inflammatory changes, as glomerular expression of ICAM-1 and VCAM-1 mRNA was upregulated by FK506 which resulted in increased macrophage infiltration. Previous reports have also shown that FK506 increases ROS production and angiotensin II expression. We identified that FK506 increased ROS production in glomeruli, especially in glomerular endothelial cells via a direct effect, which is a novel finding. The glomerular injury induced by FK506 was reduced by co-treatment with the angiotensin II receptor blocker telmisartan. These results may lend support to the view that telmisartan exerts a protective effect on the glomerular endothelium.

We have shown that FK506 increases ROS production in the rat glomerulus. Previously, Han et al. [15] reported that FK506 induces ROS production in cultured mesangial cells. In the present study, we have demonstrated that FK506 also induces ROS production in glomerular endothelial cells. The results of several studies have strongly suggested that the production of ROS is a common mechanism of FK506 toxicity. Khanna et al. [22] reported that superoxide mediates renal damage in FK506-induced...
nephrotoxicity in a rat transplant model. Furthermore, Zhou et al. [23] reported that hydrogen peroxide is important for FK506-induced toxicity in renal cells in vitro. These results are compatible with our findings.

In most cases of FK506 nephropathy, increased blood pressure causes adverse effects on renal function. Since our aim was to evaluate the direct effects of FK506 while excluding the effect of hypertension, we used a dosage of FK506 which does not affect blood pressure, and we used a similar dosage of telmisartan. Glomerular injury, such as increased numbers of adhesion molecules and infiltration of macrophages, was observed in FK506 nephropathy. However, there was no significant difference in proteinuria between experimental groups. Most likely, urinary protein which passed through the glomerulus was reabsorbed by the proximal tubule because tubular injury is not severe at the early stage in FK506 nephropathy.

NAD(P)H oxidase is a major source of ROS production in endothelial cells [24]. We have identified that FK506 activates NAD(P)H oxidase activity and increases the NAD(P)H oxidase cytosolic components p47phox and p67phox in the glomerulus. Khanna and Pieper [22] also reported that upregulation of the NAD(P)H oxidase components gp91phox and p22phox was related to production of ROS. In contrast to our results, they reported that membrane components were increased by FK506, but, in agreement with our findings, they showed that NAD(P)H oxidase activity was increased. However, the mechanism of FK506-induced upregulation of NAD(P)H oxidase components is not clear and should be explored in future studies. With regard to the mechanism of increased NAD(P)H oxidase activity, there have been some reports of how FK506 might activate enzyme activity. FK506 has been shown to promote dissociation of FK binding protein 12 (FKBP12) from the ryanodine receptor complex and to increase Ca2+ release through ion channels [25]. When FKBP12 is dissociated from the IP3R complex by FK506, calcineurin is also dissociated [26]. Therefore, IP3R is phosphorylated by protein kinase C, and release of Ca2+ through the receptor is increased [27]. These changes in intracellular Ca2+ concentration lead to activation of NAD(P)H oxidase. This mechanism may also be involved in FK506-induced endothelial superoxide production.

There are multiple sources of ROS production, such as COX, eNOS, and mitochondria, with the exception of NAD(P)H oxidase. Gp91tat, a NAD(P)H oxidase inhibitor, almost completely inhibited FK506-stimulated NAD(P)H oxidase activity in vitro. Indeed, FK506-induced ROS production in endothelial cells is mainly a result of NAD(P)H oxidase. We also performed an additional lucigenin assay experiment where we added L-Arg and xanthine as substrates. However, ROS production was not increased in these cases (data not shown). We have considered that NAD(P)H oxidase is a major source of ROS in glomeruli. However, we do not consider that all FK506-induced ROS production in glomeruli is a result of NAD(P)H oxidase because the present study is an in vitro study.

We have shown that FK506 upregulates ICAM-1 and VCAM-1 mRNA expression in the rat glomerulus. Papaccio et al. [28] reported overexpression of adhesion molecules, namely, increased ICAM-1 mRNA in islets, in FK506-treated mice. This finding suggests that FK506 could upregulate expression of adhesion molecules in several tissues. The results of a number of studies have indicated that ROS induce adhesion molecules in endothelial cells. Qin et al. [29] have reported that expression of ICAM-1, VCAM-1, and E-selectin was stimulated through NF-κB and p38 MAPK signaling pathways via the induction of ROS. Furthermore, Kim et al. [30] also demonstrated that ICAM-1 and VCAM-1 expression was regulated by ROS-dependent NF-κB activation. From these reports, it is considered that intraglomerular ROS induced by FK506 resulted in the upregulation of expression of inflammatory adhesion molecules in glomerular endothelial cells.

We have demonstrated that FK506-induced nephropathy is suppressed by telmisartan treatment. Telmisartan decreased FK506-induced NAD(P)H oxidase activity, ROS production, ICAM1 expression, and macrophage infiltration in glomeruli. Previously, we reported that ARB inhibits ROS production induced by NAD(P)H oxidase, and ameliorated endothelial dysfunction in diabetic nephropathy [16]. In the present FK506-induced nephropathy model, the AT1 receptor expression was increased in glomeruli, so it means upregulation of RAS activity. Therefore, the ARB telmisartan shows the protective effects in this FK506 nephropathy model. However, the mechanism of RAS activation by FK506 is not clear at the present time, so it should be clear in the future. On the other hand, the hemodynamic effects of ARB might also be related to protecting against glomerular injury. To evaluate such a postulation, the use of a ROS inhibitor is necessary. This topic warrants further research.

Another possible mechanism of NAD(P)H oxidase activation is the PPAR-γ-related pathway, in which PPAR-γ negatively regulates NAD(P)H oxidase activity [31]. Our findings also suggest that the inhibiting effect of NAD(P)
H oxidase activity by telmisartan mediates PPAR-γ activation. We previously reported that pioglitazone, a PPAR-γ agonist, inhibits the expression of the NAD(P)H oxidase component and decreases NAD(P)H oxidase activity [32], suggesting that telmisartan has a mechanism similar to that of pioglitazone. Telmisartan has a unique property that activates PPAR-γ; therefore, it is likely that this activation contributes to its renoprotective effects. Takeda et al. [33] reported that AT1R expression was suppressed by PPAR-γ activation. We have shown that glomerular AT1R expression is decreased by telmisartan treatment, and this mechanism of telmisartan may also contribute to RAS inhibition in FK506-treated glomeruli.

**Conclusion**

The present findings indicate that oxidative stress induced by FK506 in glomerular endothelial cells is involved mainly in NAD(P)H oxidase activity and subsequently induces inflammatory changes. This action could play a role in vascular injury and in complications associated with the long-term use of FK506. In addition, we have shown that telmisartan exerts renoprotective effects against FK506-induced nephrotoxicity via antioxidative activity. In the case of long-term treatment with FK506, we should consider that endothelial dysfunction might develop. Telmisartan might also be effective for endothelial protection from FK506-induced vascular injury.

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**Disclosure Statement**

The authors declare no conflicts of interest with regard to the study.

**References**

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