

Diltiazem minimizes tubular damage due to FK506-mediated nephrotoxicity following ischemia and reperfusion in rats

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Abstract: We examined the nephrotoxicity of tacrolimus (FK506) in a model of mild warm ischemia. After clamping of both renal arteries of male Sprague-Dawley rats for 20 min, the animals received tacrolimus (3 mg/kg/day i.p.), vehicle of a combination of tacrolimus (3 mg/kg/day i.p.) and diltiazem (12 mg/kg, orally) or vehicle and diltiazem (12 mg/kg, orally). The excretion of urinary enzymes was determined on a daily basis, creatinine clearance at day 10. Tacrolimus significantly increased NAG (*N*-acetyl- β -glucosaminidase) excretion and associated histological damage, finally decreasing creatinine clearance. The toxic potential of tacrolimus was markedly enhanced by ischemia. The additional application of diltiazem reduced NAG excretion and histological damage without affecting creatinine clearance. Thus, the protective effect of diltiazem on tacrolimus-induced nephrotoxicity seems to be at least partially a tubular one.

Introduction

FK506 (tacrolimus) is a new immunosuppressive agent in the field of transplantation. Tacrolimus binds to an intracellular *cis-trans*-isomerase, FK-binding protein (FKBP). This complex interacts with the calcium-dependent calcineurin-calmodulin complex thereby disrupting signalling in T lymphocytes.¹ In this respect, the mechanism of action is similar to cyclosporine A, which binds to a different family of isomerases, the cyclophilins.

Recent studies documented the efficacy of tacrolimus in the primary therapy following kidney and liver transplantation as well as in the treatment of acute rejection episodes, even in cases where other immunosuppressives were ineffective.² As with cyclosporine A, however, tacrolimus is potentially nephrotoxic, limiting its usefulness in kidney transplantation. It has been demonstrated that ischemia is a potentiating cofactor in cyclosporine-induced nephrotoxicity.³ The influence of ischemia on tacrolimus nephrotoxicity has not yet been determined.

Based on the intracellular mechanisms of tacrolimus, calcium-dependent mechanisms may be important during the development of tacrolimus-related nephrotoxicity. Previous studies demonstrated that the calcium-channel inhibitor diltiazem brings about not only functional, but also morphological, improvement in tacrolimus-induced acute nephrotoxicity.⁴ Diltiazem is also effective in the prevention of ischemic damage.⁵

Objective

We evaluated the effects of diltiazem upon tacrolimus-mediated nephrotoxicity in a model of mild warm ischemia resembling the conditions of transplantation.

Material and methods

Male Sprague-Dawley rats (200–250 g) were used throughout the experiments. Free access to water was given.

Under anesthesia with pentobarbital (60 mg/kg, i.p.), both renal arteries were clamped for 20 min—the average warm ischemia time in renal transplantation. For 10 days, rats were

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either treated with tacrolimus (3 mg/kg/day i.p.), a combination of tacrolimus (3 mg/kg/day i.p.) and diltiazem (12 mg/kg/day, orally), a combination of vehicle and diltiazem (12 mg/kg/day, orally) or vehicle alone ($n = 15$ per group). Twenty-four hour urine samples were collected on a daily basis. At the end of the collecting period, the urine was centrifuged and eluted using a Sephadex G-50 find column. The following enzymes were determined in the eluate using standard methods: *N*-acetyl- β -glucosaminidase (NAG, lysosomal), leucine aminopeptidase (LAP, brush border), and lactatedehydrogenase (LDH, cytoplasmic), corresponding to minor, medium and severe tubular damage, respectively. Enzyme excretion is given as units/l and expressed as U/g creatinine. At the end of the study, creatinine clearances (Cl_{cr}) were determined (ml/min/kg body weight) and the kidneys were harvested, fixed in buffered formalin, stained with hematoxylin-eosin and periodic acid-schiff (PAS) and evaluated histologically. ANOVA and Student's *t*-test were used for statistical analysis.

Results

Urinary enzyme excretion

In the vehicle group, ischemia-induced tubular injury was mild; NAG excretion peaked at day 2 postoperation and decreased slowly thereafter (Figure 1). If diltiazem was administered, this mild injury was further reduced to the levels observed in vehicle-treated animals without ischemia. Tacrolimus, on the other hand, accelerated tubular injury, even more so if ischemia and tacrolimus were combined. Again, diltiazem

reduced the amount of NAG excretion. No differences in either LAP or LDH excretion were observed in either group.

Creatinine clearance (Cl_{cr})

In the vehicle group 10 days postoperation Cl_{cr} was 8.8 ± 1.2 ml/min/kg (Table 1). No significant change was observed when warm ischemia or diltiazem were applied additionally. Treatment with tacrolimus, on the other hand, significantly decreased Cl_{cr} ($p < 0.01$), even more so if ischemia was added. However, in contrast to the findings for NAG excretion, the additional application of diltiazem did not reverse the decreased Cl_{cr} in the tacrolimus-treated groups.

Histology

In both kidneys of rats treated with vehicle and those treated with the combination of vehicle and ischemia, no tubulointerstitial or glomerular lesions were apparent as determined by light microscopy. In the tacrolimus group with additional ischemia, however, histological examination revealed isolated

Table 1 Creatinine clearance (ml/min/kg) 10 days postoperation

Group	Mean	SEM
Vehicle	8.8	1.2
Vehicle and ischemia	8.7	1.1
Vehicle, ischemia and diltiazem	8.6	1.3
Tacrolimus	6.8	1.0
Tracrolimus and ischemia	6.0	0.8
Tacrolimus, ischemia and diltiazem	6.2	0.9

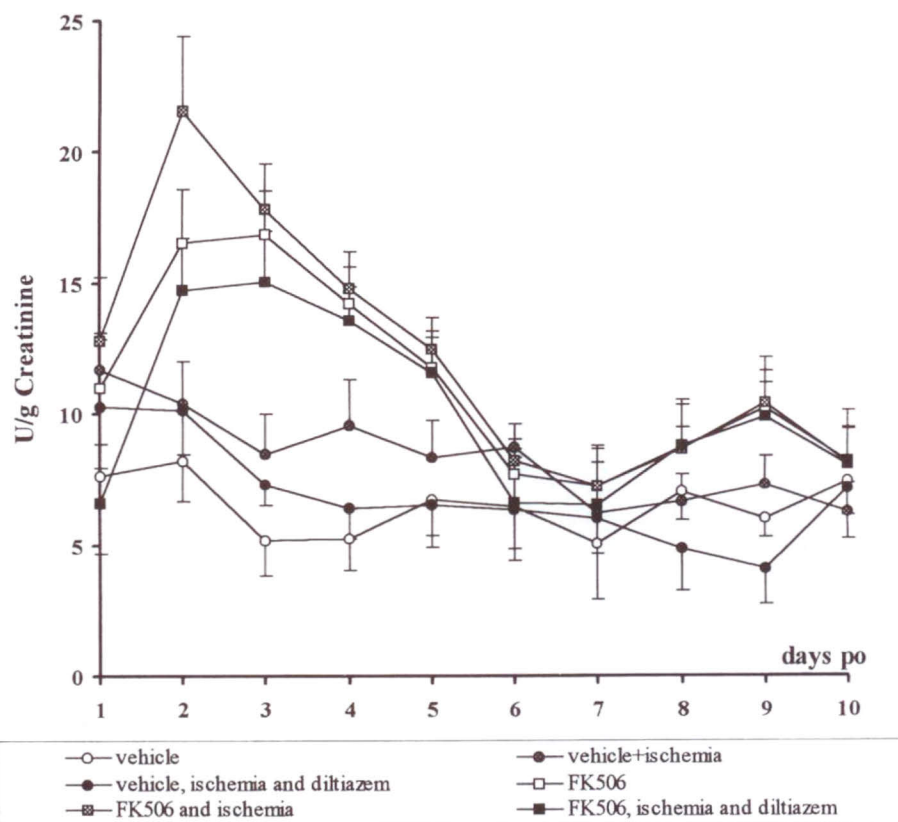


Figure 1 NAG excretion in 24-h urine. In all vehicle groups NAG excretion was low. NAG levels were highest in the tacrolimus and ischemia group; additional application of diltiazem significantly reduced NAG excretion.

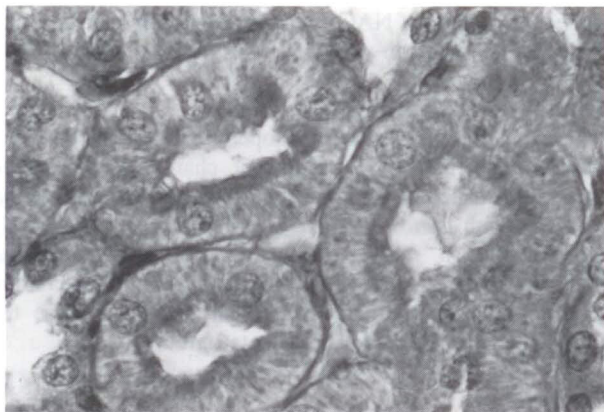


Figure 2 Kidney treated with vehicle and ischemia. There are no histological signs of tubular damage.

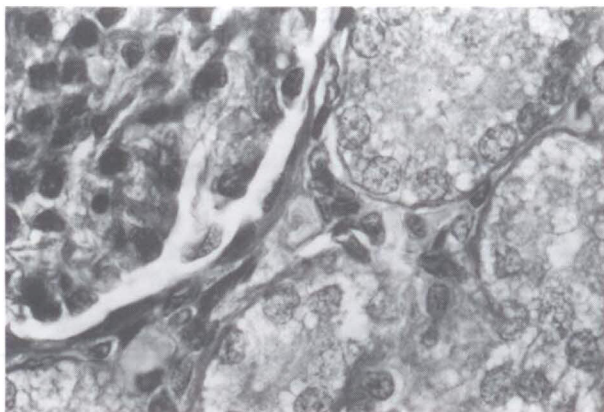


Figure 3 Kidney treated with tacrolimus and ischemia. Severe intracellular vacuolizations were present in tubular cells.

and focal degenerative tubular lesions associated with intracellular vacuolization and interstitial fibrosis (Figures 2–4). In kidneys of animals treated with the combination of tacrolimus, ischemia and diltiazem, only minor intracellular vacuolization and interstitial fibrosis were observed.

In summary, the combination of ischemia and tacrolimus proved to be more nephrotoxic than tacrolimus alone. The damage was partially reversed by diltiazem.

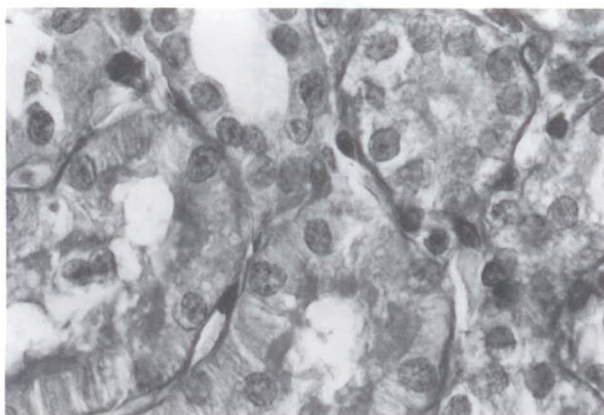


Figure 4 Kidney treated with tacrolimus, ischemia and diltiazem. Minor vacuolizations were observed in tubular cells.

Table 2 Histological assessment 10 days postoperation

Group	Vacuolization	Interstitial fibrosis
Vehicle	0	0
Vehicle and ischemia	0	0
Vehicle, ischemia and diltiazem	0	0
Tacrolimus	++	+
Tacrolimus and ischemia	+++	+++
Tacrolimus, ischemia and diltiazem	++	+

Scores: 0, none; +, mild; ++, medium; +++, severe.

Discussion

In this study we evaluated the effect of mild warm ischemia as a cofactor in tacrolimus nephrotoxicity. As tacrolimus is used primarily in transplantation, we used a model resembling kidney transplantation as closely as possible. As previously described,⁶ warm ischemia caused minor injuries as demonstrated by an increased NAG excretion without histologically apparent damage. Ischemia alone did not alter creatinine clearance. The NAG excretion reached control values 6 days after ischemia. Diltiazem prevented NAG excretion in the vehicle/ischemia group without improving Cl_{cr} . NAG values in this group did not differ from control values. The protective effect of diltiazem against ischemia/reperfusion injury seems to be related to vascular and tubular mechanisms. On the one hand, diltiazem presents vasospastic reactions following reperfusion⁷; these vasospastic reactions result from the intracellular accumulation of calcium in vascular smooth muscle cells during ischemia. On the other hand, it is known that diltiazem prevents ischemia-induced tubular necrosis.⁸

Tacrolimus significantly increased NAG excretion associated with histological damage and a decreased Cl_{cr} . The toxic potential of tacrolimus was markedly enhanced by ischemia.

FK506-binding protein (FKBP) has been previously described.⁹ This complex inhibits the action of calcineurin, the predominant calmodulin-binding protein in T lymphocytes. Calcineurin, however, is found not only in T lymphocytes but also in kidney and the nervous system.¹⁰ As calcineurin is inactivated by tacrolimus, this drug presumably increases the Ca^{2+} influx into tubular cells. This may be the reason for the inhibition of protein synthesis and cell proliferation that has been described for tubular cells.¹¹

It has also been suggested that tacrolimus-associated nephrotoxicity is correlated with tacrolimus-induced reduction in renal plasma flow and a subsequently decreased glomerular filtration rate.^{12,13} These hemodynamic alterations are thought to be the sequel to a contraction of mesangial cells induced by an increase in intracellular calcium concentration. This increase was reversed by the application of verapamil. Verapamil, like diltiazem, is an inhibitor of the type L voltage-dependent calcium channel.¹⁴

In contrast, the additional application of diltiazem to tacrolimus, although improving tubular lesions histologically, did not reverse the decrease in Cl_{cr} in this study. Thus, the protective effect of diltiazem on tacrolimus-induced nephrotoxicity seems to be at least partially a tubular one. The pathological Ca^{2+} influx into the cells may play an important role in

ischemia/reperfusion injury as well as in tacrolimus-induced nephrotoxicity, explaining the potentiation of nephrotoxicity by mild warm ischemia. The inhibition of pathological Ca^{2+} influx induced by tacrolimus and ischemia, may be the reason for the protective effect of diltiazem. Furthermore, diltiazem did not influence vascular changes induced by tacrolimus in this model. The meaning of the reduced Cl_{cr} under tacrolimus treatment remains elusive.

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