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A role for leukotrienes in cyclosporine nephrotoxicity

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Background. Nephrotoxicity associated with cyclosporine A (CsA) administration is characterized by marked renal vasoconstriction, interstitial fibrosis, and arteriolar hypertrophy. While the molecular mechanisms of CsA toxicity are not well characterized, previous studies have demonstrated that altered arachidonic acid (AA) metabolism plays a role its pathogenesis. Using a rat renal transplant model, the purpose of this study was to examine the effects of CsA on the 5-lipoxygenase (5-LO) pathway of AA metabolism.

Methods. The PVG (RT1c) strain of rats underwent kidney transplantation, and recipients of nonrejecting kidney transplants were treated with either 50 mg/kg/day CsA or vehicle ($N = 24$). To determine the physiologic significance of increased leukotriene (LT) production, the peptidoleukotriene receptor antagonist SKF 106203 was administered to CsA-treated animals for six days.

Results. CsA caused a substantial reduction in glomerular filtration rate (GFR) in the transplanted rats compared with the vehicle-treated controls (1.5 ± 0.6 vs. 4.1 ± 0.8 mL/min/kg, $P < 0.05$). The reduction in renal function was associated with enhanced urinary excretion of the peptidoleukotriene metabolites LTE_4 (1431 ± 207 vs. 953 ± 125 pg/24 h, $P < 0.05$) and N-acetyl-LTE_4 (4411 ± 848 vs. 463 ± 70 pg/24 h, $P < 0.001$). LT receptor blockade had a significant protective effect on renal transplant function in CsA-treated animals (GFR, 4.8 ± 1.1 vs. 1.7 ± 0.9 mL/min/kg, $P < 0.05$), such that CsA-treated animals that received SKF106203 maintained GFR at levels similar to controls that never received CsA (4.1 ± 0.8 mL/min/kg). Peptidoleukotriene receptor blockade also prevented the histomorphological abnormalities caused by CsA, including tubular vacuolization.

Conclusions. These studies identify a critical role for LTs in the pathophysiology of CsA nephrotoxicity and suggest that LT antagonists may be useful in preventing CsA-associated kidney toxicity.

The introduction of cyclosporine A (CsA) into clinical use has dramatically altered the field of solid organ transplantation. For example, the widespread use of CsA was associated with significant improvements in one-year and long-term renal allograft survival [1, 2]. Additionally, incorporation of CsA into immunosuppressive protocols has increased the success and scope of transplantation of the liver, heart, lung, bone marrow, and pancreas [3]. Because of its efficacy in transplantation, CsA is now being used as clinical therapy in a variety of immunologic diseases, in which it shows promise, including glomerulonephritis, psoriasis, uveitis, and inflammatory bowel disease [3–5]. Unfortunately, nephrotoxicity continues to be an important clinical entity, often limiting the therapeutic applications of CsA [6–11]. Acute CsA nephrotoxicity is characterized by diminished glomerular filtration rate (GFR) and intense renal vasoconstriction [8]. These hemodynamic alterations are dose dependent and reversible with reductions of CsA dosage. In contrast, chronic nephrotoxicity, which is characterized by vascular lesions and striped interstitial fibrosis, is irreversible and may lead to progressive renal failure and end-stage renal disease [9, 10].

The molecular mechanisms that cause CsA nephrotoxicity have not been completely characterized. However, alterations in sympathetic nerve activity, the renin-angiotensin system, and endothelin have all been implicated in the pathogenesis of this disorder [12–19]. In addition, studies from our group and others have demonstrated abnormalities in arachidonic acid (AA) metabolism caused by CsA and have suggested a role for eicosanoids in nephrotoxicity [20–24]. For example, CsA-induced alterations in the cyclooxygenase pathway of AA metabolism leading to enhanced production of thromboxane A_2 (TXA_2) have been well-documented [24, 25]. However, little is known about the effects of CsA on other pathways for AA metabolism, such as the 5-lipoxygenase (5-LO) pathway, which is shown in Figure 1. Similar to TXA_2, leukotrienes (LTs), which are produced by the 5-LO pathway, are potent renal vasoconstrictors, mimicking the hemodynamic effects of CsA [26, 27]. The objective of this study was to further define the role of 5-LO products of AA in CsA nephrotoxicity, using a rat kidney transplant model. In these studies, we find that CsA causes a marked and specific enhancement of peptidoleukotrienes in the kidney, and we provide evidence that these lipid mediators contribute to abnormalities of kidney structure and function.

Key words: 5-lipoxygenase pathway, arachidonic acid, peptidoleukotriene receptor blockade, tubular vacuolization, kidney toxicity.

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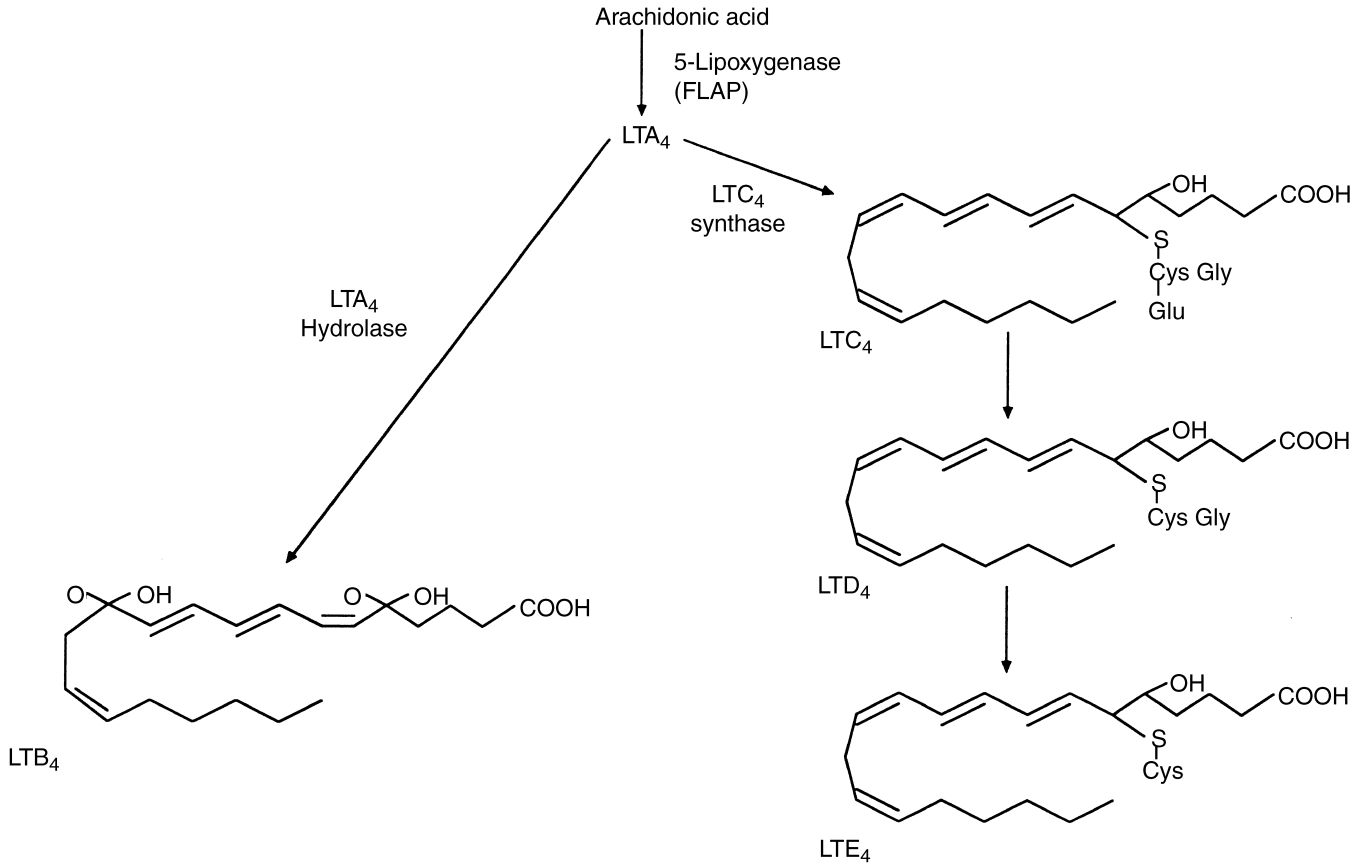


Fig. 1. The 5-lipoxygenase (5-LO) pathway of arachidonic acid (AA) metabolism.

METHODS

Animals

Inbred male rats of the PVG (RT1c) strain were purchased from Bantin and Kingman (Fremont, CA, USA). These animals were subsequently maintained in the Durham VAMC Animal Facility under local and National Institutes of Health guidelines. All rats were 8 to 12 weeks old when the studies were performed.

Rat renal transplantation

Rat renal transplants were performed as previously described [16]. For this nonrejecting isograft model, kidneys from PVG rats were transplanted into their litter mates. The donor kidney, ureter, and bladder were harvested en bloc, including the renal artery with a 3 mm aortic cuff and the renal vein with a 3 mm vena caval cuff. These vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively. The total ischemic time averaged 10 to 15 minutes. To create the urinary anastomosis, donor and recipient bladders were attached dome-to-dome. The right native kidney was removed at the time of transplant, and the left native kidney was removed through a flank incision 48 to 72 hours later. Overall, surgical mortality was less than 10% and did not differ between the experimental groups.

Drug administration

To induce CsA nephrotoxicity, CsA was administered daily by gavage at 50 mg/kg in an olive oil emulsion, as we have described previously [23]. Vehicle control groups received an equal amount of olive oil daily. In a second set of experiments that were designed to study the effects of a LT antagonist on CsA nephrotoxicity, CsA-treated animals also received the peptidoleukotriene antagonist SKF 106203 (3(S)-[(2-carboxyethyl)thio]-3-[2-(8-phenyloctyl)phenyl] propanoic acid) at 80 mg/kg or vehicle twice daily by gavage. This dose has previously been demonstrated to block the renal peptidoleukotriene receptor effectively [28]. In addition, we have previously demonstrated that the antagonist has no effect on renal function in normal rats [28] or mice [29].

Measurements of renal transplant function

To assess renal isograft function precisely, clearances of inulin and paraaminohippuric acid (PAH) were measured on day 6 following transplantation, as previously described [28, 30]. On the day of study, the animals were

Table 1. Effects of cyclosporine (CsA) and peptidoleukotriene receptor blockade on renal function and leukotriene production

Experimental group	GFR	Renal plasma flow	Renal LTB_4 production	Urinary LTE_4 excretion	Urinary N-acetyl LTE_4 excretion
	ml/min/kg		*pg/mg protein*	*pg/24 h*	
Vehicle	4.1 ± 0.8	11.5 ± 2.2	88 ± 44	953 ± 125	463 ± 99
CsA *50 mg/kg/day*	1.6 ± 0.9[a]	5.6 ± 2.0[a]	56 ± 36	1431 ± 207[c]	4411 ± 848[c]
CsA + peptidoleukotriene receptor blocker (SKF 106203)	4.7 ± 1.1[b]	13.7 ± 3.4[b]	Not measured	Not measured	6774 ± 747[c]

[a] $P < 0.05$ vs. Vehicle
[b] $P < 0.05$ vs. CsA
[c] $P < 0.005$ vs. Vehicle

anesthetized with Nembutal (20 mg/kg), and a polyethylene catheter (PE-240) was inserted into the trachea to facilitate spontaneous respiration. The right carotid artery was cannulated to permit periodic sampling of arterial blood and measure arterial blood pressure (BPM-8802; Caldwell Systems, Rougemont, NC, USA). The jugular vein was cannulated, and a volume of normal saline equal to 1.0% of body weight was infused to replace surgical losses. Following saline infusion, priming doses of ^{14}C-inulin and glycyl-^{3}H-PAH (Amersham, Arlington Heights, IL, USA) in saline were given followed by a continuous infusion at a rate of 0.05 mL/100 g/min. The ureter of the transplant kidney was cannulated with a PE-10 catheter. After a 45-minute equilibration period, urine was collected for two consecutive 30-minute clearance periods. Arterial blood samples were obtained at the midpoint of each urine collection. Tritium and ^{14}C radioactivity were measured in blood and urine with a dual-channel Isocap 300 liquid scintillation counter (Nuclear Chicago, Chicago, IL, USA). Clearances of inulin and PAH were calculated by standard formulas.

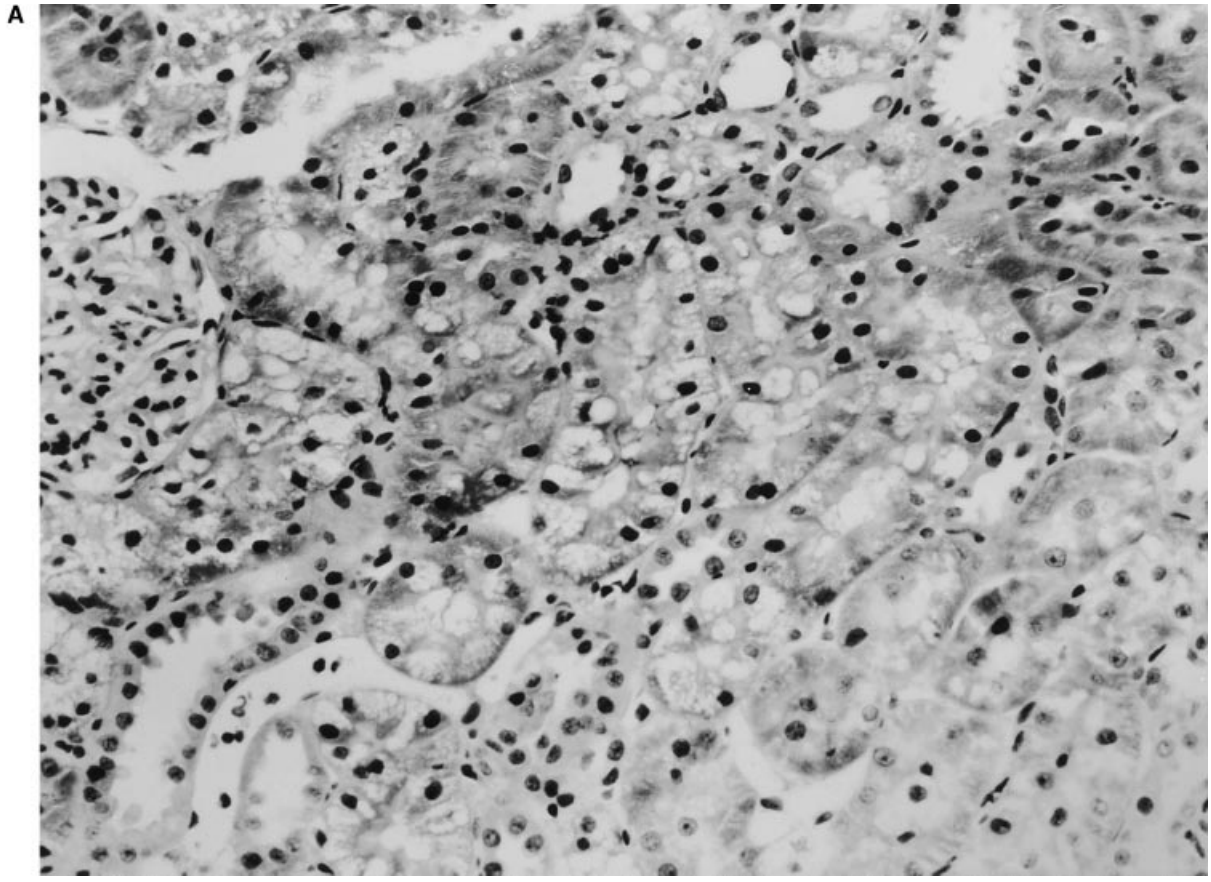
Evaluation of kidney transplant histomorphology

Following the renal hemodynamic studies, a portion of the transplanted kidney was removed and placed in 10% buffered formalin. After formalin fixation, the kidney tissue was sectioned and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stains. The slides were reviewed by a renal pathologist (P.R.) who was masked to the treatment groups and graded as described previously [30]. The severity of the vascular, tubular, and interstitial abnormalities were each graded separately using a semiquantitative scale from 0 to 4+, where 0 was no abnormality, and 4+ represented the most severe abnormality. A total score was calculated by adding the individual scores.

Measurements of leukotriene production

On day 4 following transplantation, rats were placed in metabolic cages, and urine was collected over 24 hours in a cooling chamber. At the end of the collection period, the urine was frozen and stored at −70°C until LT concentrations were measured. LT metabolites in urine were extracted and separated by high-performance liquid chromatography (HPLC) prior to quantitation by radioimmunoassay (RIA) as described previously [28], with some modifications. Samples were acidified and applied to C-18 columns preconditioned with methanol and water. After washing, the samples were eluted with a 70% solution of acetonitrile in water and evaporated to dryness under nitrogen and then reconstituted in a 1:1 solution of acetonitrile and 2.5 mmol/L trifluoroacetic acid. To separate LTs, a Pecosphere HS3-C18 cartridge (Perkin-Elmer Corp., Norwalk, CT, USA) was used. Samples and LT standards (Advanced Magnetics Inc., Cambridge, MA, USA) were injected onto the column and eluted with a linear gradient from 100% 2.5 mmol/L trifluoroacetic acid to 100% acetonitrile over eight minutes at a flow rate of 3 mL/min. Elution of LTs from the column was monitored by absorbance at wavelength 280 nm with a programmable multiwavelength ultraviolet spectrophotometer (Waters Associates, Milford, MA, USA). In this HPLC system, the LT standards for LTE_4 and its major urinary metabolite, N-acetyl-LTE_4, were resolved in two closely eluting absorbance peaks. All separations were performed at ambient temperature. Collected fractions were evaporated to dryness under nitrogen and reconstituted in appropriate buffer for quantitation by RIA,

Fig. 2. Effects of peptidoleukotriene receptor blockade on renal histopathological changes in rats treated with cyclosporine A (CsA). (*A*) Representative photomicrograph of an HE-stained section of a renal isograft from a rat that was treated with CsA 50 mg/kg/day for six days. The major structural abnormality in these kidneys is diffuse vacuolization of proximal tubule cells that can been seen throughout the section. (*B*) A similarly stained section of a renal isograft from a rat that received CsA along with the peptidoleukotriene receptor antagonist SKF106203. Kidney histomorphology was virtually normal in these animals and not significant from that of renal isografts from animals that never received CsA (data not shown).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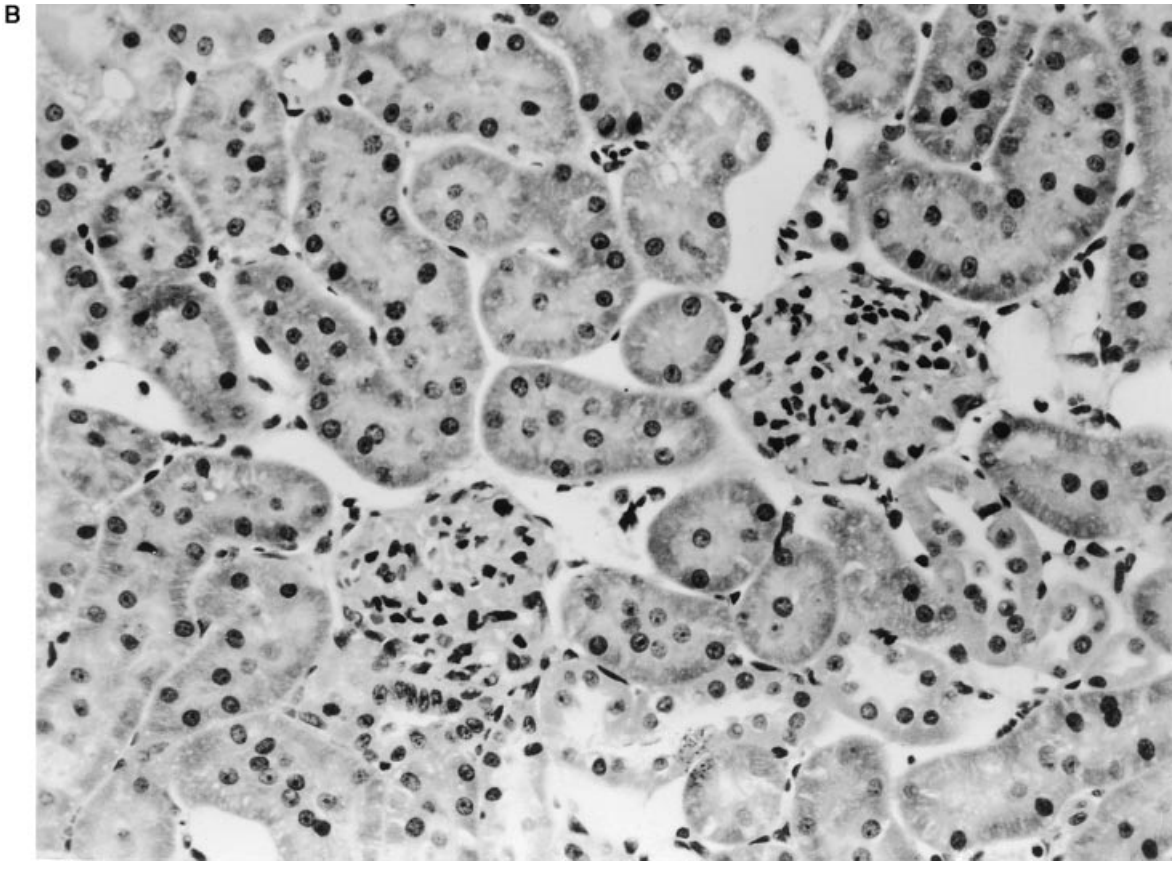
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as described later in this article. Using this technique, recovery of standards ranged from 40 to 60%.

Following renal hemodynamic studies, the transplant kidney was rapidly removed, and a portion was weighed and placed in Krebs buffer at 4°C. The kidney was then bisected, and a central slice was obtained. Cortex was separated from medulla by macrodissection. Portions of cortex were homogenized and suspended in 2 mL of iced Kreb's buffer containing 10 μmol/L concentrations of the calcium ionophore A23187. The suspensions were incubated for 30 minutes at 37°C in 95% O_2/5% CO_2. Samples were centrifuged at 2000 r.p.m. for 10 minutes at 4°C, and the supernatants were stored at −70°C until eicosanoids were measured, as described later in this article. The tissue pellet was resuspended in 0.5 mL of cold Kreb's buffer, and the protein concentration was measured using the Coomassie Brilliant Blue dye-binding assay.

Radioimmunoassays

Concentrations of LTB_4 in supernatants from incubations of kidney homogenates were measured by direct RIA as previously described [28] using a polyclonal antisera (Amersham). Samples (measured in duplicate) and standards (measured in triplicate) were incubated with known quantities of [^{3}H]-LTB_4 and antisera at 25°C for two hours. The concentration of unlabeled LTB_4 that inhibited 50% binding of [^{3}H]LTB_4 tracer was 42.7 pg/100 μL (mean standard error of 4 assays), and the lower limit of delectability was 0.5 pg/100 μL. For measurement of peptidoleukotriene excretion in urine, individual HPLC fractions were collected based on elution of authentic LTE_4 and N-acetyl-LTE_4 standards. Concentrations of urinary LTE_4 and N-acetyl-LTE_4 in experimental specimens were then measured using a polyclonal antibody that cross-reacts with a series of peptidoleukotrienes, including LTE_4 and N-acetyl-LTE_4 [18]. The concentration of unlabeled LTE_4 that inhibited 50% binding of [^{3}H]LTE_4 tracer was 284 ± 19 pg/100 μL (mean standard error of 3 assays), and the lower limit of delectability was 8 pg/100 μL. After incubation for two hours at 4°C, unbound eicosanoids were removed from the mixture with a suspension of dextran-coated activated charcoal. Sample concentrations were determined by a standard curve in which the logarithm of the concentration is plotted versus the log of the B/Bo value. Results were expressed as pg/min/mg protein. We found that the cross-reactivity of the LTB_4 antibody (Amersham) was less than 1% with LTC_4, LTD_4, and LTE_4. Cross-reactivity of the polyclonal peptidoleukotriene antibody with LTB_4 was also less than 1%.

Statistical analysis

Data are presented as mean ± SEM. For the hemodynamic studies, data points for each animal represent the mean of the values measured during at least two clearance periods. For most comparisons between groups, statistical significance was assessed using an unpaired *t*-test. The Mann–Whitney *U*-test was used in the comparisons of the histopathologic scores.

RESULTS

The effect of CsA administration on renal isograft function on day six after transplantation is shown in Table 1. CsA caused marked reductions in clearances of inulin and PAH. In this model, CsA treatment reduced GFR by approximately 60% (1.6 ± 0.9 mL/min/kg in the CsA-treated group vs. 4.1 ± 0.8 mL/min/kg in vehicle-treated controls, $P < 0.05$); renal plasma flow (RPF) was reduced to a similar extent (5.6 ± 2.0 mL/min/kg with CsA vs. 11.5 ± 2.2 mL/min/kg in controls, $P < 0.05$).

As an index of renal LT production in vivo, we measured urinary excretion of LTE_4 metabolites. As shown in Table 1, CsA produced a dramatic, nearly tenfold increase in excretion of N-acetyl LTE_4 metabolites from 463 ± 99 pg/24 h in vehicle-treated animals to 4411 ± 848 pg/24 h in the rats that received CsA ($P < 0.005$). An increase in excretion of LTE_4 was also observed. We also examined the effect of CsA on the production of $LT B_4$ in cortical homogenates following stimulation with A23187. Unlike its effect on the peptidoleukotrienes, CsA administration had no significant effect on renal production of LTB_4 (56 ± 36 vs. 88 ± 44 pg/mg protein/min, P = NS).

To test whether augmented LT production contributes to reduced renal function in CsA-treated animals, rats with renal isografts were also given 50 mg/kg/day of CsA along with 80/mg/kg/day of the specific peptidoleukotriene receptor antagonist SKF 106203 or its vehicle. As shown in Table 1, in the group that received vehicle, CsA significantly reduced renal isograft function. In contrast, treatment with the peptidoleukotriene antagonist preserved GFR at levels that were not different from isograft controls not treated with CsA. The LT receptor antagonist had a similar beneficial effect on RPF. These effects appeared to be related to blockade of the peptidoleukotriene receptor and not to an alteration in CsA metabolism or inhibition of peptidoleukotriene production, as the peptidoleukotriene receptor antagonist did not prevent the enhanced excretion of N-acetyl LTE_4 associated with CsA administration (Table 1).

To determine the effect of CsA on histomorphology in renal isografts, structural abnormalities were examined and graded by a pathologist (P.R.) who was masked to the treatment groups. As can be seen in the representative photomicrographs shown in Figure 2, the primary histologic abnormality observed in animals treated with CsA was marked tubular vacuolization. This abnormality has been previously reported to be a characteristic fea-

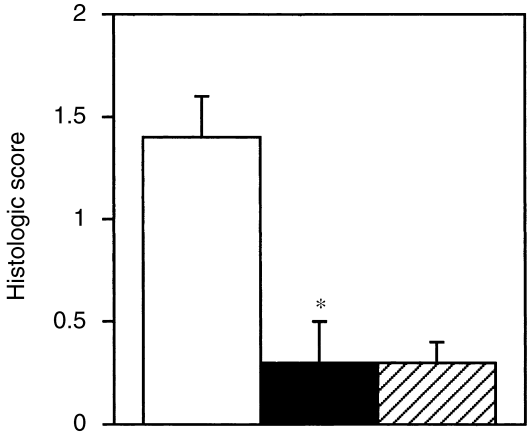

Fig. 3. Histopathological grading of rat renal isograft sections. The severity of the histopathological abnormalities observed in renal isografts from the experimental groups were graded as described. The degree of histopathological abnormality was significantly reduced in the group that received CsA + SKF 106203 (■) compared with the group that received CsA alone (□). Moreover, there was no significant difference in histopathological score between the group that received CsA + SKF 106203 and the group that received olive oil vehicle without CsA (▨) (*$P < 0.01$).

ture of CsA toxicity in the rat [31]. To determine whether improved renal function seen with peptidoleukotriene receptor blockade was associated with changes in kidney histology, we compared the histomorphology of renal isografts from the CsA-treated rats that received SKF106203 or its vehicle. As illustrated in the photomicrographs in Figure 2, renal architecture was virtually normal in the animals that received SKF106203. The significant degree of tubular vacuolization that was seen in the vehicle group was not observed in the group that received the LT-receptor antagonist. As shown in Figure 3, these differences were reflected by a significant reduction in the semiquantitative scoring for histologic abnormalities in the SKF106203 group (0.3 ± 0.2) compared with controls (1.4 ± 0.2, vs. SKF106203). The histologic grade of kidney grafts from CsA-treated animals that also received SKF106203 was not different from kidneys in animals that had never received CsA.

DISCUSSION

Alterations in the renal sympathetic nervous system, the renin angiotensin system, endothelin, and nitric oxide have all been implicated in the pathogenesis of CsA nephrotoxicity [12–19, 32, 33]. In addition, we and others have previously demonstrated that alterations in synthesis of the AA metabolite TXA_2 may mediate renal hemodynamic actions of CsA [20–24]. For example, in a rat model, CsA nephrotoxicity is accompanied by enhanced production of thromboxane metabolites by the kidney [24]. We subsequently found that the administration of a thromboxane receptor antagonist partially abrogated renal dysfunction in this model [23]. Perico et al demonstrated a similar effect of a thromboxane synthase inhibitor in rats with CsA nephrotoxicity [20]. In renal transplant patients treated with CsA, we found that urinary excretion of thromboxane metabolites was enhanced. Additionally, treatment with a thromboxane synthase inhibitor produced transient increases in RPF and GFR in 8 of 10 patients [34]. Thus, in animal models and in human renal transplant recipients, dysregulation of the cyclooxygenase pathway of AA metabolism contributes to the pathogenesis of CsA nephrotoxicity.

We now provide evidence that abnormal production of 5-LO metabolites of AA causes kidney dysfunction and injury in CsA nephrotoxicity. As shown in Figure 1, AA that is released from membrane phospholipids by phospholipases is converted by 5-LO into LTA_4. The activity of 5-LO in intact cells is dependent on 5-LO activating protein or FLAP. LTA_4 is further metabolized by one of two pathways: It is converted to LTB_4 by the enzyme LTA_4 hydrolase, or it is metabolized along the peptidoleukotriene pathway, beginning with the addition of glutathione to form LTC_4 by the enzyme LTC_4 synthase. This divergence of LTA_4 metabolism has functional significance. LTB_4 is a potent chemotactic agent that promotes neutrophil aggregation, adhesion and enzyme release [27], while the peptidoleukotrienes (LTC_4, LTD_4, LTE_4) are potent vasoactive substances [26, 27, 35]. Infusions of LTC_4 reduce GFR by increasing arteriolar vasoconstriction and diminishing the glomerular ultrafiltration coefficient [26]. These hemodynamic effects of peptidoleukotrienes mimic the effects of CsA on renal hemodynamics. Furthermore, in cell culture models, peptidoleukotrienes stimulate synthesis of collagen and extracellular matrix proteins [36, 37]. The abnormal accumulation of collagen and matrix proteins in the renal interstitium is one of the defining features of chronic CsA nephrotoxicity.

In a rat renal transplant model, we find that the administration of CsA causes a marked stimulation of peptidoleukotriene production, reflected by enhanced excretion of peptidoleukotriene metabolites in urine. The administration of CsA results in an exclusive stimulation of peptidoleukotriene production; LTB_4 production is not significantly affected. This suggests a direct effect of CsA on the expression and/or activity of LTC_4 synthase. Alternatively, stimulation of LT production by CsA may be confined to cell types that primarily synthesize peptidoleukotrienes and not LTB_4. In support of this latter hypothesis are observations by our group and others that the administration of CsA is associated with a significant accumulation of macrophages in the kidney [23, 38, 39]. Activated macrophages synthesize large quantities of LTC_4, but very little LTB_4 [27].

A physiological role for enhanced peptidoleukotriene production in CsA toxicity was established by the obser-

vation that the administration of the peptidoleukotriene receptor antagonist SKF 106203 completely prevented the reduction in GFR and RPF caused by CsA. In addition, this treatment also abrogated the development of proximal tubule vacuolization that is characteristic of CsA nephrotoxicity in rodents. Thus, in this model that was designed to mimic the effects of CsA in a transplanted kidney without the confounding effects of rejection, we find a critical role for peptidoleukotrienes in the pathogenesis of renal dysfunction and structural changes in the kidney. We conclude that the beneficial effects of LT receptor blockade in this model are due to interruption of the effects of peptidoleukotrienes to cause renal vasoconstriction and to alter glomerular permeability [26]. Our data suggest that LTs also promote the development of renal morphological changes and proximal tubular vacuolization. However, the mechanism of these effects is not apparent from our experiments. The development of vacuoles in tubular epithelium in rats with CsA nephrotoxicity was first described by Thomson et al, but the basis of these abnormalities is not known [31].

Studies by Perico et al had first suggested a role for peptidoleukotrienes in CsA nephrotoxicity [21]. These investigators found that the administration of a single bolus of CsA into the renal artery caused immediate reductions in GFR and RPF that could be partially prevented by administration of a peptidoleukotriene receptor antagonist. Treatment with the TXA_2 receptor antagonist GR32191 had a similar effect, consistent with previous studies showing modest, but significant effects of thromboxane inhibitors in CsA nephrotoxicity [20, 22, 23, 34]. When the LT and thromboxane receptor antagonists were given together, the hemodynamic effects of CsA were completely blocked, leading the authors to conclude that both TXA_2 and peptidoleukotrienes contribute to acute renal vasoconstriction caused by CsA [21]. Our study supports a causative role for LTs in mediating the renal hemodynamic effects of CsA and extends these results into an in vivo model of renal transplantation. However, our data are consistent with a dominant role for peptidoleukotrienes in this model.

In conclusion, in a model of CsA nephrotoxicity in rat renal isografts, the daily administration of CsA reduced renal function by approximately 50% and caused renal structural abnormalities. Blockade of peptidoleukotriene receptors prevented the functional and structural effects of CsA, suggesting that peptidoleukotrienes play a key role in the pathogenesis of CsA nephrotoxicity in this model. Based on these findings and prior studies demonstrating the efficacy of LT antagonists in preventing renal dysfunction associated with allograft rejection, we speculate that peptidoleukotriene antagonists may be useful adjunctive therapy in transplant patients that are treated with CsA.

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