Nitric Oxide Production Modulates Cyclosporin A-Induced Distal Renal Tubular Acidosis in the Rat

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ABSTRACT

Cyclosporine A (CsA) causes distal renal tubular acidosis (dRTA) in humans and rodents. Because mice deficient in nitric oxide (NO) synthase develop acidosis, we examined how NO production modulated H⁺ excretion during acid loading and CsA treatment in a rat model. Rats received CsA, l-arginine (L-Arg), or N⁴-nitro-L-arginine methyl ester (L-NAME), or combinations of CsA and L-NAME or L-Arg, followed by NH₄Cl (acetic acid load). In vehicle-treated rats, NH₄Cl loading reduced serum and urine (HCO₃⁻) and urine pH, which was associated with increases in serum [K⁺] and [Cl⁻] and urine NH₃ excretion. Similar to CsA (7.5 mg/kg), L-NAME impaired H⁺ excretion of NH₄Cl-loaded animals. The combination CsA and L-NAME reduced H⁺ excretion to a larger extent than either drug alone. In contrast, administration of L-Arg ameliorated the effect of CsA on H⁺ excretion. Urine pH after NH₄Cl was 5.80 ± 0.09, 6.11 ± 0.13*, 6.37 ± 0.16*, and 5.77 ± 0.09 in the vehicle, CsA, CsA + L-NAME and CsA + L-Arg groups, respectively (*P < 0.05). The effect of CsA and alteration of NO synthesis were mediated at least in part by changes in bicarbonate absorption in perfused cortical collecting ducts. CsA or L-NAME reduced net HCO₃⁻ absorption, and, when combined, completely inhibited it. CsA + L-Arg restored HCO₃⁻ absorption to near control levels. Administration of CsA along with L-NAME reduced NO production to below levels observed with either drug alone. These results suggest that CsA causes dRTA by inhibiting H⁺ pumps in the distal nephron. Inhibition of NO synthesis may be one of the mechanisms underlying the CsA effect.

Cyclosporine A (CsA) is a potent, widely used immunosuppressive agent. Unfortunately, this agent has major adverse reactions (Bennet, 1983; Bennet et al., 1996) and renal toxicity limits its clinical application. Although acute renal failure due to vasoconstriction of the renal arteriole is well known (Bennet et al., 1996), CsA-related renal tubular acidosis is also clinically important (Battie et al., 1986; Stahl et al., 1986; Heering et al., 1996), because the acidosis might affect the compensatory growth of transplanted organs. CsA-related renal tubular acidosis is distal type in both humans (Stahl et al., 1986) and animals (Jaramillo-Juarez et al., 2000); however, the mechanism(s) for the effect(s) of CsA on tubular function is unknown.

CsA induces vasoconstriction of the renal artery by reducing production of nitric oxide (NO) (De Nicola et al., 1993; Bobadilla et al., 1994). In fact, l-arginine, a substrate for NO synthase, improves the drug-induced vasoconstriction and tubular fibrosis (Assis et al., 1997; Zhang et al., 1999). On the other hand, there is evidence that NO inhibits H⁺-ATPase activity in the cortical collecting duct (CCD) (Tojo et al., 1994). Based on these latter data, treatment of the CsA-induced hypertension with l-arginine could aggravate the distal renal tubular acidosis (dRTA). For these reasons, we examined whether renal dRTA induced by CsA is affected by changes in the level of NO; l-arginine was used as a donor for NO, and NO production was reduced by administering nitro-l-arginine-methyl ester (l-NAME), an NO synthase inhibitor. Results of this study demonstrate that increased NO production ameliorates the dRTA due to CsA and that impaired NO production may contribute to the impaired rate of H⁺ excretion induced by CsA.

Materials and Methods

Animals and Treatments. Male Wistar rats (8 weeks old, n = 110) were used in this study. They were maintained in a specific pathogen-free room with free access to standard rat chow (CE-2, containing 0.3 g/100 g of Na⁺, 1.06 g/100 g of K⁺, and 1.18 g/100 g of Ca²⁺; Japan Clea Co., Ltd., Tokyo, Japan) and distilled water. Temp...
temperature and humidity were automatically controlled in the room. All the experiments were conducted in accordance with the Jichi Medical School Guideline for Laboratory Animals. Cyclosporine A (7.5 mg/kg; Shihab et al., 2000) or vehicle (olive oil) was given to rats by oral gavage once a day in the morning for 6 weeks. L-NAME (12.5 mg/kg for low dose and 30 mg/kg for high dose), L-arginine (L-Arg, 8.5 mg/kg; Shihab et al., 2000) or vehicle (distilled water) was also given to these animals twice daily (every 12 h) for 6 weeks by gavage [n = 16 in each group except for the vehicle group (n = 18) and high dose of L-NAME (n = 9)]. Each agent was diluted in distilled water. The dosages of L-NAME and L-Arg were determined empirically to be the smallest effective doses that minimally inhibited weight gain. Because our preliminary study showed that the body weight in the CsA + L-NAME (high-dose) group was significantly decreased, and the reduction itself might affect acid-base status in these animals, we selected a lower dose of L-NAME for combined L-NAME + CsA treatment.

Systolic arterial blood pressure was measured by tail cuff method on a weekly basis. The dose of CsA (7.5 mg/kg) has previously been shown to not elevate the blood pressure of rats (Shihab et al., 2000).

Oral Challenge Test for Evaluation of Tubular Acidification Capacity. One of two types of oral challenge tests for the evaluation of tubular acidification capacity was performed at 2, 4, and 6 weeks after the initiation of the study. These tests were performed by a randomized, crossover design and in accordance with the diagnostic tests of human renal tubular acidosis (Rose, 1994) with slight modifications. On the day of each test, animals received NH$_4$Cl (1.6 g/kg with 3% body weight of distilled water) or vehicle (distilled water, 3% of body weight) by nasogastric tube. Thereafter, each animal was placed in a metabolic cage for a 4-h urine collection under water-saturated light mineral oil to avoid evaporation (Tsruoka et al., 2000a, 2001a). After the end of the urine collection, animals were placed in a small warm box (at 38°C) for about 10 min to dilute their veins before sampling blood. Then, the animals were placed in a small cage to avoid moving during the blood sampling.

Approximately 5 ml of blood was obtained from the tail vein by insertion of a 22-gauge needle. Part of the blood was mixed with approximately 0.05 ml of heparin for measurement of bicarbonate concentration, and the rest was allowed to clot and was centrifuged to provide a serum sample. Urine pH was measured immediately after the end of the collection. A portion of the collected urine was added with a mixture of sulfonic acid/sodium tungstate to remove protein and used for measurement of ammonia (NH$_3$). The rest was kept frozen until the measurement of electrolytes and nitric oxide.

Measurement of Ion Concentrations in Blood and Urine. Urine pH and concentrations of creatinine, Na$^+$, K$^+$, and Cl$^-$ in serum and urine were measured by an electrometer (HM-16S; Toa Electronics, Tokyo, Japan) and AutoAnalyzer (model 716S; Hitachi, Tokyo, Japan), respectively. Bicarbonate concentration was determined by an enzymatic method (diagnostic kit 132-A; Sigma-Aldrich, St. Louis, MO). Ammonia concentration was measured by Berthelot reaction (Killeen et al., 1993). Urinary nitrate and nitrite concentrations (NO$_3^-$ and NO$_2^-$), stable metabolites of nitric oxide in the urine, were determined by a colorimetric assay/nitrite/nitric oxide colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI).

In Vitro Microperfusion of Isolated CCDs. In vitro microperfusion was performed according to the method of Burg et al. (1966) with minor modifications (Tsruoka et al., 1993, 2001b). A kidney was removed 2 to 3 days after the last oral challenge test. To avoid any potential tubular damage by acute NH$_4$Cl loading, only water-loaded animals were selected for tubule microdissection. A CCD was isolated from cortico-medullary ray by fine forceps under a stereomicroscope. Composition of artificial solution for bath fluid and perfusate was as follows: 115 mM NaCl, 2.5 mM K$_2$HPO$_4$, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 4 mM Na-lactate, 1 mM Na$_2$-citrate, 5.5 mM d-glucose, 6 mM l-alanine, and 25 mM NaHCO$_3$. The volume of the collecting pipette was 13.5 nl. The mean perfusion rate was 1.7 ± 0.3 nl/min (n = 42). Transepithelial voltage was measured using the perfusion pipette as the luminal electrode.

The concentration of total CO$_2$ (assumed to be equal to that of HCO$_3^-$) was measured by Nanofo (WPI, Sarasota, FL) (Tsruoka et al., 2001b; Schwartz et al., 2002). Net bicarbonate transport was calculated as J$_{HCO3}$ = (Cl$^-$ - Co)/(Vl/W), where Cl and Co are bicarbonate concentrations of collected fluid and perfusate, respectively, Vl is the rate of collected fluid, and L is length of the tubule. A positive value indicates absorption of bicarbonate (H$^+$ secretion). In each group there were eight tubules dissected from six animals.

The calculation of J$_{HCO3}$ is based on there being no net water flow across the CCD in the absence of arginine vasopressin (Gross et al., 1975). To confirm this assumption, we measured fluorescein isothiocyanate-insulin transport by nanoflo fluorometry (WPI) (Tsruoka et al., 2001b) in three CCDs isolated from normal rats. We confirmed that there is no net water transport in CCDs: Jv = -0.01 ± 0.01 nl/min/mm (not significantly different from zero). Gross leakage was checked by addition of FD&C green dye to the perfusate during the experiments (Tsruoka et al., 1993, 2000b, 2001b; Tsruoka and Schwartz, 1996).

Statistics. All the data are presented as mean ± S.E. Statistical analysis was performed by one-way analysis of variance and, if significant, the Fisher's protected least significant difference test was used to compare the groups. These analyses were done by StatView 5 for Windows (SAS Institute Inc., Cary, NC). P < 0.05 is regarded as significant.

Results

Effects of CsA, L-NAME, and L-Arg on Body Weight, Systolic Arterial Pressure, and Creatinine Clearance. In a previous study, Jaramillo-Juarez et al. (2000) reported that a higher dose of CsA (50 mg/kg) in uninephrectomized rats led to substantial weight loss as well as acidosis in the absence of changes in glomerular filtration rate (GFR) or in proximal tubular function. To reduce the potential for anorexia and increased catabolism resulting in weight loss, we used a lower dose of CsA, which did not influence weight gain or markedly affect baseline serum bicarbonate levels. Nevertheless, this lower dose of CsA (comparable with what is often used clinically in renal transplant patients) caused distal renal tubular acidosis in the rats. All the animals completed the study. Their body weights, determined twice weekly during the study, did not decrease during the treatment period, although the weight increment was greatest in the vehicle group. Because the higher dose (30 mg/kg) of L-NAME + CsA reduced body weight in preliminary studies, we used a lower dose (12.5 mg/kg) of L-NAME for concomitant use with CsA in this study. We found that even the low dose of L-NAME in conjunction with CsA substantially inhibited renal acid excretion (see below).

Systolic arterial blood pressure in drug-treated rats was not significantly different from vehicle control group with the exception of the high dose L-NAME group. Systolic arterial blood pressure at 6 weeks after the administration of the drugs was 135 ± 4, 138 ± 6, 137 ± 6, 135 ± 6, 139 ± 6, 146 ± 5*, and 134 ± 4 mm Hg for vehicle, CsA, CsA + L-NAME, CsA + L-Arg, low-dose L-NAME, high-dose L-NAME, and L-Arg, respectively (*P < 0.05).

We also measured creatinine clearance during the distilled water challenge. It was not different among the seven groups (5.7 ± 0.5, 5.3 ± 0.6, 5.0 ± 0.8, 5.5 ± 0.7, 5.3 ± 0.8, 4.8 ± 0.8, and 5.6 ± 0.7 ml/min/kg in vehicle, CsA, CsA + L-NAME, CsA + L-Arg, low L-NAME, high L-NAME, and L-Arg, respectively).
tively; n = 9 each). Assuming the creatinine clearance to be a reasonable estimate of GFR, these results indicate that the drug treatments did not markedly alter GFR.

Treatment of Rats with L-NAME Induces Renal Tubular Acidosis in Water-Challenged Rats. CsA induces distal renal tubular acidosis. To determine whether production of NO regulates distal renal acidification, we treated rats with L-NAME or L-Arg alone or in combination with CsA. Urine acidification and compensatory changes in serum electrolytes were measured. Mean urine pH in vehicle group after distilled water challenge was 7.27 ± 0.12 pH units (Fig. 1A). By statistical comparison with analysis of variance, we found that the pH was significantly different among the seven groups. Treatment of rats with CsA and high-dose L-NAME significantly lowered urine pH. The urine pH of rats treated with CsA + L-NAME (low dose) was significantly lowered but was not significantly different from rats treated with CsA alone. Whereas L-Arg alone did not affect urine pH, coadministration of CsA + L-Arg prevented the decrease in urine pH.

Urine NH₃ concentrations (Fig. 1B) were not significantly different among the seven groups. Urine HCO₃⁻ concentrations were nearly zero in all groups (data not shown). Mean serum K⁺ concentration in the group treated with vehicle was 3.71 ± 0.13 mEq/l (Fig. 1C). It was significantly different among the seven groups. Compared with vehicle group, it was significantly higher in CsA, CsA + L-NAME, and high-dose L-NAME groups. In the CsA + L-Arg group mean serum K⁺ concentration was restored to the level in the vehicle group.

Mean serum Cl⁻ concentration in the vehicle group was 98.6 ± 0.13 mEq/l (Fig. 1D). It was significantly increased in

![Fig. 1](https://via.placeholder.com/150)

Fig. 1. Urine pH (A), NH₃ (B) excretion, serum K⁺ (C), Cl⁻ (D), and bicarbonate (E) concentrations after acute loading of distilled water or NH₄Cl to rats. Animals were treated with CsA, CsA + L-NAME, CsA + L-Arg, L-NAME (low dose 12.5 mg/kg, high dose 30 mg/kg), L-Arg, or vehicle for 2 to 6 weeks. For CsA + L-NAME, the low dose of L-NAME was used because the high dose of L-NAME + CsA reduced body weight in preliminary studies. Free-voided urine was collected for 4 h in a metabolic cage just after the loading of fluid. Blood was obtained from the tail vein just after the end of the collection of urine. The two challenge tests were performed with randomized crossover fashion with 2-week intervals in all the groups.
CsA, CsA + l-NAME, and high-dose l-NAME groups. Serum Cl− was further increased in CsA + l-NAME group compared with the CsA group. Serum Cl− concentration in the CsA + l-Arg group was restored to the level in the vehicle group.

Serum HCO₃⁻ concentration was also affected by CsA and l-NAME (Fig. 1E). CsA and high-dose l-NAME reduced serum HCO₃⁻ concentration compared with the vehicle group (24.6 ± 0.03 mEq/l). Serum HCO₃⁻ was further reduced in the CsA + l-NAME group compared with the CsA group and was restored to normal in the CsA + l-Arg group. These results demonstrate that stimulation of NO production prevents, and blockade of NO synthesis exacerbates, the dRTA induced by CsA.

**CsA and/or L-NAME Inhibit H⁺ Excretion in Response to an Acid Load.** NH₄Cl loading of rats markedly reduced urine pH [NH₄Cl-loaded (5.80 ± 0.09 units) versus control (7.27 ± 0.12 units); Fig. 1A]. In groups treated with CsA or high-dose l-NAME, urine pH was significantly higher than vehicle group, but this was not observed in the low-dose l-NAME group. Coadministration of l-NAME (low dose) with CsA further increased urine pH in acid-loaded animals. In contrast, l-Arg treatment alone did not affect urine acidification, but restored urine pH to normal in acid-loaded animals that had also been given CsA.

Urine HCO₃⁻ concentrations after NH₄Cl loading were nearly zero in all the groups (data not shown). Changes in urinary NH₃, and serum K⁺, Cl⁻, and HCO₃⁻ exhibited a similar pattern: specifically, the acid challenge elevated urine NH₃ concentration and serum K⁺, Cl⁻, and HCO₃⁻ concentrations compared with water-challenged rats (Fig. 1). Administration of high-dose l-NAME or CsA decreased NH₃ excretion and serum HCO₃⁻ concentration, while increasing serum K⁺ and Cl⁻ levels in acid-loaded animals. The combination of CsA and l-NAME had a greater effect than either drug alone (Fig. 1, B–E). Treatment with l-Arg alone did not alter compensatory changes to an acid load, whereas coadministration of l-Arg with CsA restored urine NH₃, and serum Cl⁻, HCO₃⁻, and K⁺ to levels that were similar to vehicle control. These results demonstrate that an inhibitor of NO synthesis potentiated, whereas increased NO production attenuates, the negative effects of CsA on H⁺ secretion during an acid load.

**CsA and l-NAME Reduce JHCO₃⁻ by CCDs.** To determine whether the effects of CsA and l-NAME on acid secretion were mediated, at least in part, by changes in transport at the level of the CCD, JHCO₃⁻ was measured in microperfused isolated segments. JHCO₃⁻ of rat CCDs generally showed net bicarbonate absorption (Fig. 2). In CCDs taken from rats treated with CsA, JHCO₃⁻ was significantly reduced compared with animals receiving vehicle. High-dose l-NAME treatment of the animals also significantly lowered JHCO₃⁻ albeit to a lesser extent than CsA. Bicarbonate absorption was completely blunted by coadministration of CsA + l-NAME (low dose). Administration of l-Arg alone did not affect JHCO₃⁻, however, coadministration of l-Arg with CsA to the animals enhanced JHCO₃⁻ to levels that were not significantly different from vehicle. These results indicate that NO production regulates bicarbonate transport and modulates the effect of CsA on JHCO₃⁻ in the rat CCD. Transepithelial voltage was not significantly different among the groups [−1.9 ± 0.2, −2.2 ± 0.4, −2.1 ± 0.3, −1.9 ± 0.4, −1.7 ± 0.3, −1.9 ± 0.4, and −2.1 ± 0.4 in vehicle, CsA, CsA + l-NAME, CsA + l-Arg, l-NAME (low), l-NAME (high), and l-Arg groups, respectively].

**Inhibition of Nitrate Excretion by CsA and l-NAME.** The similarity between the effects of CsA and l-NAME on H⁺ secretion suggests that CsA may cause dRTA through inhibition of NO production. To test this hypothesis, levels of urinary nitrate in nanomoles per microgram of creatinine were determined as an indirect measure of NO production in rats. Administration of l-Arg markedly increased (vehicle control 0.27 ± 0.02 versus l-Arg 1.29 ± 0.20), and conversely, l-NAME (high dose) significantly reduced urinary nitrate/creatinine ratios (Fig. 3). These data suggest that levels of urinary nitrate reveal information about systemic changes in NO production. CsA did not significantly reduce nitrate levels despite a numerical decrease (P = 0.08); however, CsA reduced urinary nitrate when administered in conjunction with low-dose l-NAME, to levels that were significantly lower than either the low dose l-NAME or CsA alone (Fig. 3). The latter results are consistent with the observations presented in Fig. 1, showing that CsA and l-NAME have additive effects on dRTA, and renal H⁺ excretion.
Discussion

Nephrotoxicity limits the use of CsA as a therapeutic agent (Bennet, 1983; Bennet et al., 1996). dRTA, which is accompanied by hyperkalemia and hyperchloremia, is a serious adverse effect in patients with organ transplantation (Stahl et al., 1986; Heering and Grabensee, 1991) and idiopathic uveitis (Aguilera et al., 1992) and in animals (Jaramillo-Juarez et al., 2000) treated with CsA. In the clinical situation, RTA is diagnosed from the urinary findings obtained from oral NH4Cl challenge tests (Rose, 1994); however, there are few studies that evaluate in vivo tubular function in animal models (Jaramillo-Juarez et al., 2000). In our study, rats were placed in a metabolic cage for 4 h after loading with water or acid (NH4Cl), so that adequate urine volume (≥5 ml) was available to help evaluate renal tubular function (Tsuruoka et al., 2000a, 2001a). The results presented herein demonstrate that similar to the effects of CsA, inhibition of NO production induces dRTA, and inhibits H+ excretion during an acid load. Moreover, this study demonstrates that elevation of NO production reverses, and inhibition of NO synthesis exacerbates dRTA and blunting of H+ excretion by CsA. The effects of CsA, the NO donor (l-Arg) and the NO synthase inhibitor (l-NAME) were mediated at least in part through changes in HCO3 transport in the rat CCD.

CsA treatment resulted in dRTA with hyperkalemia in this study, which differs from the more common type 1 dRTA in humans that is accompanied by hypokalemia (Rose, 1994). dRTA patients with hyperkalemia may have either 1) a voltage defect due to diminished distal Na+ transport and thereby impaired excretion of H+ and K+ ions (hyperkalemic type 1 dRTA), or 2) aldosterone deficiency or resistance (type 4 RTA) (Rose, 1994). Urine pH after acid loading of patients with the hyperkalemic type 1 RTA humans is usually above 5.5, whereas in the type 4 RTA patients it falls below pH 5.5 (Rose 1994). Although our vehicle-treated rats did not consistently acidify the urine below pH 5.5 and did not receive a trial of mineralocorticoids to treat type 4 RTA (Rose, 1994), nevertheless, based on the analysis of their responses to water and NH4Cl, those receiving CsA and l-NAME had a much smaller reduction in urine pH than did the vehicle-treated rats (Fig. 1A), suggesting the equivalent of a hyperkalemic type 1 dRTA. On the other hand, CsA treatment is generally considered to cause a type 4 RTA (Rose, 1994). Further studies will be needed to determine whether NO and CsA can reduce aldosterone secretion, which results in a type 4 RTA (Rose, 1994).

Reduced NO production is implicated in the mechanisms not only for vasoconstriction and tubular fibrosis (Andoh et al., 1997; Assis et al., 1997) but also for extrarenal complications of CsA (Orji and Keiser, 1998; Fiore et al., 2000). Assis et al. (1997) and Zhang et al. (1999) also reported that l-arginine improves renal function in transplant patients with CsA. Results of this study are consistent with the supposition that CsA induces renal dRTA through inhibition of NO production. Although our low dose of CsA alone did not significantly reduce systemic NO production, CsA in combination with a low dose of l-NAME significantly reduced NO production (Fig. 3). Shihab et al. (2000) reported findings similar to our results, whereas Assis et al. (1997) reported an increase in nitrate excretion with CsA. We are not able to explain the results of Tojo et al. (1994) who found that NO inhibited the proton pump in rat CCDs. With regard to the referenced studies, species and conditions of the experiments, such as water loading, might account for some of the variability of the results. Because some of the effects of CsA on NO production may be localized to the renal vasculature or tubular segments, conclusive demonstration of CsA’s effects on NO production in the kidney awaits further study using isolated tissues or cells.

The effects of l-Arg and l-NAME reported in this study are consistent with a previous study demonstrating a role for endothelin-1 and endothelin-B receptors in H+ excretion. Endothelin-1 is produced by renal tubule epithelia (Wesson, 1997; Laghimani et al., 2001) and plays a pivotal role in renal adaptation to acidosis. Metabolic acidosis increases endothelin-1 addition to the renal cortical interstitial fluid (Wesson, 1997), which helps mediate the renal adaptation. Wesson (1997) showed by in vivo microperfusion that inhibition of endothelin-B (ET-B) receptors during chronic acid loading blunted the enhanced rate of proton secretion. Activation of ET-B receptors leads to enhanced NO production through tyrosine kinase- and Ca2+/calmodulin-dependent pathways (Tsukahara et al., 1994). In lung cells, acidosis has also been found to stimulate NO production (Pedoto et al., 1999). Because CsA inhibits calcium-dependent signal transduction in selected cell types (Pedoto et al., 1999), one can speculate that CsA inhibits ET-B receptor-mediated regulation of NO synthase activity. Indeed, cyclosporin has been found to inhibit NO production in a variety of cell types (Orji and Keiser, 1998; Harris et al., 2001; Lima et al., 2001). In addition, knockout mice deficient in neuronal nitric-oxide synthase exhibit a metabolic acidosis (Wang et al., 2000), clearly indicating an important role for NO in the transport of H+ in vivo.
tecture studies are directed toward determining which type of intercalated cell is affected by CsA treatment. Our present studies indicate that NO is involved in the adaptation, especially of α- or β-type intercalated cells in response to metabolic acidosis. This finding may be useful for understanding the mechanism by which CsA induces dRTA and may provide the basis for ameliorating this problem in the future.

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References

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