Effects of Cyclosporine and Its Metabolites in the Isolated Perfused Rat Kidney

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ABSTRACT

The isolated perfused rat kidney (IPK) was used to study the acute effects of cyclosporin A (CsA) and its metabolites (M1, M17, M18, M21 and M-COOH). GFR, renal vascular resistance, and sodium, potassium and water reabsorption were measured before and after the addition of CsA/metabolites/vehicles. There was no difference in CsA effect (mild decrease in GFR and increase in renal vascular resistance with the inclusion of plasma (10 ml) or whole blood (20 ml) in the albumin perfusate (120 ml). Intralipid was used as the vehicle for CsA and the metabolites because methanol, ethanol, and Cremophor had significant effects on GFR. Intralipid enhanced the effect of CsA 25-fold, giving CsA dose responses comparable to those of human kidneys. This enhanced effect with Intralipid was due to vasoconstriction, not vascular obstruction, and was apparently specific to CsA (no enhancement of norepinephrine with Intralipid). The primary metabolites (M1, M17, and M21) caused decreases in GFR comparable to or slightly less than those caused by CsA. The secondary metabolites (M18 and M-COOH) caused more modest declines in GFR. Cyclosporine metabolite levels in patient blood often greatly exceed levels of the parent drug; these studies suggest that the metabolites may contribute significantly to CsA nephrotoxicity in patients.

Key Words: Cyclosporin A, cyclosporine metabolites, Intralipid, isolated perfused kidney, nephrotoxicity

Cyclosporin A (CsA) is a potent immunosuppressive agent without myelotoxicity. Its use in transplant patients has greatly improved the survival of allografts but has been complicated by acute and chronic nephrotoxicity. CsA has also been shown to be effective in treating a number of other immune-mediated disorders, but its use has been limited by potential nephrotoxicity. Despite much study of the mechanisms of CsA nephrotoxicity, they are still not clearly defined, and it is not known whether the toxic effect is due solely to CsA or to its metabolites and vehicles, as well. Several forms of CsA nephrotoxicity have been described; these include: (1) acute, reversible vasoconstriction, which, if severe enough, may result in acute renal failure; (2) proximal tubule pathology, which seems to occur at CsA levels above 1.500 to 2.000 ng/mL, but not at therapeutic levels; (3) renal vascular injury, an inconsistent clinical finding; and (4) chronic, irreversible interstitial fibrosis, which seems to occur only after 6 to 12 months of CsA therapy (1) in patients subjected to high blood concentrations of CsA (2). Most of the evidence to date suggests that CsA nephrotoxicity is dose dependent (2), and great care is taken in clinical settings to maintain blood levels of CsA in a narrow target range (100 to 250 ng/mL) to avoid potential nephrotoxicity (3).

CsA is a neutral cyclic peptide that is extremely hydrophobic, and it therefore has the capacity to interact with biologic and synthetic phospholipid bilayer membranes. This characteristic also requires that CsA be dissolved in a nonpolar vehicle, such as lipid or alcohol, in order to be solubilized/emulsified in aqueous solutions. The iv form of CsA has a Cremophor-ethanol vehicle: the oral forms, used for the chronic administration of CsA, are in olive oil or corn oil. Cremophor EL is a nonionic solubilizer and emulsifier obtained by reacting ethylene oxide with castor oil, producing a glycerol-polyethylene glycol ricinoleate. The LD₅₀ for iv administration in the mouse is 2.5 to 4 mL/kg (BASF, Parsippany, NJ). Both ricin and ethylene glycol are potent nephrotoxins, so there would seem to be at least the potential for nephrotoxicity with Cremophor.

The CsA metabolites M1, M17, M18, and M21 and the carboxy-metabolite M-COOH all retain the decapeptide ring structure of the parent compound. They are produced by various substitutions of groups around the ring, and metabolism occurs primarily by the cytochrome P-450 system in the liver. The majority of the metabolites (90%) are excreted in bile, with normally a small percentage (6%) entering the circulation and being excreted in urine (4). Hepatic im-

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pairment reduces metabolite elimination via the biliary system and increases the amounts of metabolites entering the circulation. Patients receiving CsA therapy may have blood metabolite levels far higher than levels of the parent drug (3,5), and marked increases in metabolite levels often immediately precede and/or coincide with increases in serum creatinine thought to be due to CsA nephrotoxicity (3,5; L.M. Shaw, unpublished data). This is intriguing circumstantial evidence for a possible role of metabolites in nephrotoxicity, but to date, there has been no direct evidence that the metabolites contribute to nephrotoxicity.

The acute effects of CsA have been studied in the isolated perfused rat kidney (IPK) in order to avoid the complications of systemic toxicity and nonrenal metabolites. Recent studies in the IPK have documented a decrease in GFR when CsA was added to the perfusate (6-8); however, two of the three studies showed comparable GFR decreases with the vehicles alone (6,8). The GFR decrease was seen when CsA was given in its SANDOZ (Basle, Switzerland) iv vehicle (Cremophor and ethanol) (6,7) or in methanol (8). There has been conflicting evidence, however, as to whether Cremophor itself is toxic. Besarab et al. reported that Cremophor with methanol was at least as toxic as CsA with methanol, and they suggested that CsA and Cremophor had a synergistic effect (8). Sumpio showed that Cremophor alone had no effect (7). Luke et al. found that Cremophor and ethanol had the same effect as CsA, Cremophor, and ethanol (8). These three studies are difficult to interpret because of the complications inherent in the use of multiple vehicles, the effects (synergistic or opposite) of which confound the effect of CsA itself. Ethanol, which is 33% of the SANDOZ iv CsA vehicle, has been shown to be a potent vasoconstrictor in the IPK (9). Methanol appeared to cause significant vasodilatation in the studies of Besarab et al.; the differences compared with control values were not statistically significant, but the data are strongly suggestive of a methanol effect (8). Only one of the studies to date used Cremophor alone and found no effect (7). The other two studies showed a "Cremophor" effect, but the Cremophor was combined with methanol (8) or ethanol (6).

Another confounding aspect of these studies was perfusate composition. Besarab et al. used perfusate that contained blood; Luke et al. and Sumpio used no blood. Kidneys perfused with blood behave differently from those perfused with albumin alone, and this difference also may have affected the results. Another discrepancy between investigators has been in the dose of CsA required to produce a given effect in the IPK. Sumpio found a greater decrease in GFR with 500 ng/mL (7), than Besarab et al. found with 1,000 ng/mL (8); Luke et al. found no greater effect at 50,000 ng/mL than with vehicle alone (6). It is difficult to explain such discrepancies in dose response, but they may be due, at least in part, to differences between investigators in terms of perfusate composition, vehicles, CsA binding to the apparatus, CsA measurements, or batches of albumin.

There are a number of questions that arise from these studies of acute CsA toxicity. Does perfusate composition have an effect on the expression of CsA toxicity in the IPK model? Because it seems that all other vehicles tested to date have some effect on the IPK, is there another vehicle that has no effect on IPK function that could be used to deliver CsA into an aqueous solution? Do the metabolites of CsA have any effects on the function of the IPK?

In the studies reported here, three aspects of CsA toxicity in the IPK were examined: first, the effect of perfusate constituents; second, the effect of vehicles; and third, the possible contribution of CsA metabolites to nephrotoxicity.

METHODS

Male Sprague-Dawley rats (300 to 400 g) were given Purina Chow (Purina Mills, St. Louis, MO) and water ad libitum. Rats were anesthetized with ip thiobutabarbital (Inactin; Byk Gulden, Konstanz, Germany) at a dose of 150 mg/kg. The right kidney was surgically removed as described by Nishihitsuishi-Uwo et al. (10); there is no interruption of flow to the kidney with this procedure. Heparin (400 U) was given iv immediately before renal artery cannulation. In studies where whole blood was added to the perfusate, 20 mL of blood was drawn from the aorta into a heparinized syringe immediately after the cannulation of the renal artery. Each kidney was studied for five consecutive 10-min clearance periods 25 min after the placement of the kidney on the perfusion apparatus. Perfusion pressures were maintained at 100 mm Hg at the tip of the cannula. Perfusion flow rate, GFR, and urine production were expressed per gram wet weight of the contralateral kidney. GFR was measured from the clearance of \[^{3}H\]lulin (11,12). GFR in the isolated kidney is usually slightly less than that in vivo, and it is influenced by many factors, including the perfusate composition (albumin quality and quantity, amino acids, blood constituents), the blood gas levels, the cleanliness of the apparatus, and the surgical skill and experience of the investigator. The IPK is unstable immediately after removal from the rat. Despite uninterrupted renal arterial flow, the initial 20 to 30 min on the perfusion apparatus is characterized by vasoconstriction, low GFR, and low urine flow rates. By 30 min after transfer to the apparatus, however, GFR and all other parameters have improved and reach their peak between 30 and 40 min. After 40 min of

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perfusion, there is a slow and steady decline in function over the next several hours. The mean GFR was expected to reach a peak of 0.7 to 1.0 mL/min·g (13–15), and any kidney not reaching 0.5 mL/min·g was excluded from the study. This was an extremely rare occurrence and almost always due to renal vascular anomalies that resulted in unperfused zones.

All kidneys were perfused with media containing 7 g/dL of BSA (Fraction V; Pentex; Miles Inc., Kane- kakee, IL) in Krebs-Henseleit bicarbonate (KHB) buffer containing sodium (145 to 155 mEq/L), potassium (4.8 to 5.8 mEq/L), chloride (105 to 115 mEq/L), bicarbonate (23 to 25 mEq/L), calcium (10 to 13 mg/dL), PO₄ (3.2 to 4.4 mg/dL), and magnesium (2.5 to 4.0 mg/dL). Glucose (125 to 150 mg/dL) and amino acids (1 mL Trophamine 6%; Kendall-McGaw, Irvine CA) were added before the perfusate was filtered. In experiments with rat blood, 20 mL of heparinized blood was added to the perfusate 5 min after it was withdrawn from the rat kidney donor. Pooled normal human serum, which had been stored at −20°C, was used in the serum studies (10 mL/120 mL of perfusate). CsA was used in its iv form (Sandimmun, 50 mg/mL in Cremophor and ethanol) or as powder (provided by Sandoz, Basel, Switzerland). CsA vehicles used to distinguish CsA effects from vehicle effects included polyoxyethyalted castor oil (Cremophor EL, provided by BASF Corp.), Sandimmun "placebo" (Cremophor 66%–ethanol 33%, vol/vol, provided by Sandoz), absolute methanol (Fisher HPLC grade; Fisher Scientific, Pittsburgh, PA) and Intra- lipid (iv fat emulsion, 20%; Kabivitrum Inc, Alameda, CA). Metabolites were provided by Sandoz: M₁ and M₂₁ were synthesized by Wenger (16); M₁, M₁₇, M₁₈, and M-COOH were prepared by Bollinger et al. and purified by silica gel chromatography (16; R.M. Wenger, personal communication). Purity (97%) was determined by HPLC, nuclear magnetic resonance spectroscopy, and gas chromatography–mass spectrometry.

The perfusate was prepared by the dissolution of 1 kg of albumin in 8 L of KHB (12.5 g/dL). This solution was dialyzed by a Lundia Minor (Gambro, Lund, Sweden) parallel plate dialyzer (pediatric size) against 13 L of KHB for 7 h at 5°C. The dialysate was changed once during the 7-h period. The perfusate was pumped through the dialyzer at a pressure of 90 mm Hg to maintain the albumin concentration at 12.5 g/dL. The perfusate thus prepared was aliquoted and kept frozen at −20°C. Just before a perfusion, 70 mL of the perfusate was thawed, glucose and amino acids were added, and the volume was brought to 120 mL with KHB. An additional 500 μL of 1 M NaHCO₃ was added to bring the bicarbonate concentration to 24 mEq/L. The perfusate was filtered through two 0.45-μm-pore-size filters (Nalgene; Nalge Co., Rochester, NY), 60 mL through each filter unit, and was imme-

diately added to the perfusion apparatus. The perfusate was gassed with a carbon dioxide–oxygen mixture, which resulted in pH 7.45 ± 0.05; Pco₂, 36 to 40 mm Hg; and Po₂, 325 to 425 mm Hg. The gas mixture came from separate oxygen and carbon dioxide tanks attached to a Bird oxygen blinder. A blender setting of 11% CO₂–89% O₂ at 3.5 L/min gave the perfusate gas values above. Gas exchange and warming took place in a Silastic (Dow-Corning) tubing membrane oxygenator. A blood transfusion filter ( Pall Biomedical Products Corp, East Hills, NY) was placed in the perfusion circuit, and the perfusate was recirculated and maintained at 37.0 ± 0.05°C, as measured in the insulated reservoir beneath the kidney. The perfusate was warmed, gassed, and recirculated 20 to 30 min before the kidney was placed on the apparatus.

Perfusate and urine sodium and potassium were measured with ion-specific electrodes (Corning 902, Na/K analyzer; Ciba-Corning, Medfield, MA) in all samples and were used to calculate percent reabsorption. The only exception to this was the clearance periods, during which the urine volume was insufficient for assay (<30 μL/10 min), i.e., clearances where GFR approached zero. Sodium reabsorption in the IPK preparation varies depending on the perfusate constituents such as albumin, amino acids, red blood cells, serum, and other factors such as salt deprivation, GFR, etc. Mean sodium reabsorption, as reported by three different laboratories in 1975 to 1976 was 96.7, 97.2, and 98.7% (14). Under more ideal conditions, the mean sodium reabsorption was found to be 99.1 to 99.7% (SE < 0.5%) (15). Sodium reabsorption was used as an indicator of the functional integrity of the IPK in the studies reported below.

The tubing and oxygenator used for the CsA studies were used only for CsA studies, because of previous reports of CsA adherence to the tubing (6,7) and because of a desire to prevent potential cross-contamination of experiments.

CsA was measured in all perfusate and tissue samples by an RIA (specific monoclonal antibody) (3) (Sandoz). Metabolite concentrations were measured by HPLC in ether extracts of perfusate and homogenized tissue (14,15). Whole kidneys were frozen at the end of the perfusion (−20°C) and were assayed later in batches.

In studies designed to rule out the possibility that the enhancement of the CsA effect by Intralipid was the result of mechanical obstruction or thromboembolic event caused by CsA in Intralipid, a series of kidneys were perfused with CsA-Intralipid for 3 to 7 min, removed from the apparatus, and immediately placed in buffered formalin for histopathologic evaluation.

Data are given as means ± SE. A repeated measures
analysis of variance model was used to analyze the data presented in Figures 2 through 5. In this model, the effects of treatment (CsA, CsA metabolites, vehicles), time (Clearance Periods 1 through 5), and the treatment × time interactions were included. The mixed effects model required that the effect of kidney within treatment was random and that the other effects were fixed. The kidney × treatment error term was used to test for the treatment and time effects.

RESULTS

Perfusate Constituents

In order to determine whether perfusate constituents, such as serum or whole blood, had any effect on acute CsA toxicity in this model and to establish the dose of CsA at which any effect would be seen, a series of kidneys were perfused in several pilot studies. In the studies reported in Figure 1, CsA and/or vehicle (methanol) were added before the beginning of five consecutive 10-min clearance periods, in either standard albumin perfusate or in standard albumin perfusate with added serum.

Initial CsA studies were done with standard perfusate containing albumin (labeled "control" and "cyclosporine" in Figure 1). There was no evidence of a significant effect of CsA at concentrations of 40 to 1,000 ng/mL of perfusate, (2,400 to 46,000 ng/g of tissue), compared with methanol (14 mg/100 mL) vehicle, or labeled "control" (Figure 1). These CsA concentrations were produced by the addition of either 60 or 600 µg of CsA to 120 mL of perfusate. The data from these two doses of CsA were combined in Figure 1 ("cyclosporine") because there was no apparent difference between them. With the inclusion of human serum (10 mL/120 mL of perfusate), the GFR was lower during the first three clearance periods than in the standard albumin perfusate ("serum" versus "control"; Figure 1). The reason for this impaired GFR with the addition of human serum to the perfusate is not known. CsA caused a slight decline in GFR ("serum" versus "serum and cyclo-
sporine"; Figure 1) at concentrations of 800 to 1,100 ng/mL of perfusate and 35,000 to 45,000 ng/g of tissue (600 µg of CsA added to 120 mL of perfusate). This decrease in GFR was associated with a small increase in renal vascular resistance (RVR).

After these pilot studies, nine kidneys were perfused under conditions as close as possible to those of Sumpio's report (7) (data not shown). We were unable to reproduce his results and could document a GFR decline of only 25% at mean CsA concentrations of 2,900 ng/mL (versus Sumpio's reported 100% decline at 750 ng/mL).

In order to determine whether there might be some factor in whole fresh blood that would allow a greater response to CsA, a series of kidneys were studied in which 20 mL of heparinized blood was drawn from the rat immediately after renal artery cannulation and was added to the perfusate 5 min later (Figure 2). CsA powder (3 or 0.3 mg) was dissolved in 100 µL of methanol and was added to the perfusate after the second control clearance period. At mean CsA concentrations of 5,000 ng/mL and 36,000 ng/g of tissue, there was a small but significant decline in GFR (P = 0.0007). The decline in GFR at extremely high
concentrations of CsA (25,000 ng/mL; 108,000 ng/g of tissue) was still only 60%.

In all three types of perfusate (albumin alone, albumin with serum, and albumin with whole blood), the effect of CsA (in methanol vehicle) was modest and comparable. We suspect that the vasoconstrictive effect of CsA in this model may be blunted by the vasodilation caused by the methanol vehicle (see Vehicles below). The CsA dose response for GFR was less than the response reported by Besarab et al. and much less than the response reported by Sempio. These results do not provide an explanation for the differences between investigators in CsA dose response, but they do suggest that it is not due to differences in perfusate composition.

Because the overall function of the kidneys perfused with blood was superior to those perfused without (renal vascular tone, distal nephron integrity and function, and GFR were closer to normal) and because the model was thought to be closer to in vivo conditions with blood than without, blood-containing perfusate was used in all subsequent studies.

**Vehicles**

It was evident from the previous studies on perfusate composition (in which methanol was the CsA vehicle) that methanol alone caused a significant increase in GFR (Figure 2). Sandimmun "placebo" (Cremophor and ethanol) had no apparent effect on GFR (Figure 3), but Cremophor alone caused a small but significant decrease in GFR (P = 0.0001).

In attempts to find a vehicle for CsA that would not have an effect of its own, Intralipid, a 20% emulsion of soybean oil, egg yolk phospholipids, glycerin, and water, (used for parenteral nutrition) was tested in the IPK. At doses as high as 1 mL/120 mL of perfusate, Intralipid had no apparent effect on any of the IPK functional parameters measured. CsA powder, mixed with Intralipid by vortex and added to the perfusate reservoir (0.2 mg of CsA in 20 µL of Intralipid added to 120 mL of perfusate), had a profound effect on GFR (Figure 4a). This effect was immediate, occurring as soon as CsA reached the kidney. The 40-min GFR for high-dose CsA (Figure 4a) is greater than zero because there was approximately 2 min of normal urine flow before CsA reached the kidney (2-min circuit time from reservoir to kidney). The surface of the kidney became severely mottled, urine flow stopped almost completely, and renal vascular resistance increased dramatically and progressively (Figure 4g). These effects were seen with a mean CsA concentration of 2,000 ng/mL of perfusate and 90,000 ng/g of tissue. At a lower CsA concentration (600 ng/mL perfusate and 16,000 ng/g of tissue), there was a 50% decrease in GFR (Figure 4a), and a moderate increase in renal vascular resistance (Figure 4g).

In order to rule out the possibility that these effects were the result of mechanical obstruction or thromboembolic event caused by CsA in Intralipid, a series of kidneys were perfused with CsA-Intralipid for 3 to 7 min, removed from the apparatus, and immediately placed in buffered formalin for histopathologic evaluation. There was no evidence on any of the tissue sections that mechanical obstruction of the vasculature was the cause of the dysfunction observed. The glomerular capillary lumina were smaller in the CsA-treated kidneys than in the controls, suggesting that the dysfunction was due to vasoconstriction and/or decreased ultrafiltration (Kf) due to mesangial contraction.

These results indicate that CsA is 25 to 50 times more toxic in this model when the vehicle is Intralipid than when the vehicle is methanol. Cremophor, or Cremophor and ethanol, and they suggest that the way the drug is delivered is of equal or greater significance than the amount of drug delivered.

In order to rule out the possibility that this enhanced toxicity was a nonspecific effect of Intralipid that would occur in the IPK with any vasoconstricting agent, a series of kidneys were perfused with norepinephrine or norepinephrine in Intralipid. There was no difference between the two (Figure 5), suggesting that this enhancement of vasoconstriction by Intralipid was peculiar to CsA. Pilot studies (data not shown) in which the perfusate contained Intralipid, but the CsA was added in methanol vehicle, did not show the enhanced toxicity, suggesting that there is some property of the CsA-fat emulsion mixture, or perhaps some constituent of the Intralipid, that allows this profound enhancement of acute CsA effect. Further support for the theory that the way the CsA

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![Graph](image-url)

**Figure 3. Effect of CsA and its 4v vehicle on GFR. Control (N = 10), no addition. Sandimmun (the IV form of CsA, vehicle is Cremophor and ethanol (N = 6), 100 µL added, CsA 22,000 ng/mL. Sandimmun "placebo" (Cremophor and ethanol) (N = 5), 100 µL added. Cremophor (N = 5), 100 µL added. Perfusate contained 20 mL of fresh whole blood; additions after 30 min (arrow). P < 0.03 and "P < 0.0001 versus the control.
Figure 4. Effects of CsA and its metabolites on GFR and vascular resistance. Control (N = 6), 20 μL of Intralipid added to 120 mL of perfusate, 20 mL of fresh whole blood/120 mL of perfusate. Low-dose CsA (N = 5), 500 ng/mL; high-dose CsA (N = 6), 2,000 ng/mL; low-dose metabolite 17 (N = 6), 300 ng/mL; high-dose metabolite 17 (N = 3), 900 ng/mL. Low-dose metabolite 1 (N = 7), 400 ng/mL; high-dose metabolite 17 (N = 5), 4,000 ng/mL. Metabolite 21 (N = 6), 500 ng/mL; low-dose metabolite 18 (N = 6), 700 ng/mL; high-dose metabolite 18 (N = 3), 1,500 ng/mL. Carboxy-metabolite (N = 6), 700 ng/mL. Additions at 30 min (arrows). *P < 0.05 and **P < 0.001.

is presented is of more importance than the actual dose is provided by CsA measurements. In virtually all of the CsA studies, there were measurable levels of CsA in the perfusate during the control clearance periods preceding the addition of CsA. This phenomenon has been reported before [5,6] and is probably the result of the adherence of CsA to the sliastic tubing of the apparatus (despite rigorous cleaning between experiments with detergent, acid, and alkali). In some of the Intralipid-CsA studies, the postaddition CsA levels were as little as 100 to 200 ng/mL higher than the “control,” preaddition levels, and yet, a significant fall in GFR occurred after the addition. One possible explanation for this might be that CsA delivered in a fat emulsion is able to interact more completely with endothelial cell membranes, whereas only a small proportion of CsA delivered in alcohol or alcohol-lipid (or CsA leaching from tubing into an aqueous media) is able to interact with the endothelial membrane. Thus, it may be that the effective dose of CsA is increased by the use of Intralipid as the vehicle, because a greater percentage of the drug is delivered to the site of action.

CsA Metabolites

The purified metabolites were tested in the IPK with blood-containing perfusate, with Intralipid as the vehicle. Blood-containing perfusate was chosen because kidney function was superior and because the model was thought to be closer to in vivo conditions with blood than without. Intralipid was chosen as the vehicle because: (1) it had no effect on the IPK, even at doses 50 times the amount used as the vehicle in these studies; (2) it is a widely used and well-accepted iv agent; (3) it has the physical properties
Cyclosporine and Its Metabolites in the IPK

Figure 5. Effect of Intralipid as vehicle on the IPK response to norepinephrine (NOREPI). Control (N = 5), 20 µL of Intralipid added. Norepinephrine (N = 5), 8 ng/mL. Norepinephrine and Intralipid (N = 5), norepinephrine added in 20 µL of Intralipid vehicle, 8 ng/mL. Additions at 30 min (arrow).

(stable fat emulsion) that allow extremely hydrophobic compounds (CsA and its metabolites) to be added to an aqueous solution; and (4) the CsA dose response in this model, with Intralipid as the vehicle, is comparable to the doses reported to be associated with toxicity in transplant patients. The aim of these studies was to determine the effect, if any, of metabolites at doses comparable to the high- and low-dose CsA studies shown in Figure 4—a high dose sufficient to cause virtually complete cessation of filtration and a low dose that would produce an intermediate effect, roughly a 50% decrease in GFR. It was not always possible to have comparable doses of metabolite, in part because of the extremely limited supply of metabolites (e.g., no high-dose M21 because of insufficient quantity) and in part because the measured concentration of metabolite was often not the concentration predicted from the amount added. This may have been because of differences between the metabolites and CsA in the degree of "sticking" to the apparatus, or it may have been because of inaccuracies in the assay methods. We thought it best to report the means of the actual measured concentrations, rather than the concentration predicted from the amount of metabolite added. All of the metabolites caused significant reductions in GFR, some even in very low concentrations (Figure 4). The primary metabolites M1, M17, and M21 caused decreases in GFR comparable to, or slightly less than, those caused by CsA. Of the metabolites tested, M21 appeared to have the greatest effect, with a 50% reduction in GFR at a mean concentration of 500 ng/mL of perfusate (Figure 4d). M17 was almost as potent as M21, causing a 66% reduction in GFR at 900 ng/mL (Figure 4b). M1 caused a 21% reduction in GFR at 400 ng/mL and a 90% reduction at 4,000 ng/mL (Figure 4c). M-COOH caused a 30% reduction in GFR at 500 ng/mL (Figure 4f), and M18 caused a 30% reduction in GFR at 1,500 ng/mL (Figure 4e). In this model, as in all other models (including humans), CsA toxicity seems to be dose dependent, and the doses of CsA and metabolites that produce significant toxicity in this model are comparable to those reported to be associated with toxicity in transplant patients.

It seems that the metabolites may have a different mechanism of action than the parent drug. M21 and high-dose M17 both caused increases in vascular resistance (Figure 4h and j), but they were very slight (did not reach above the control) and seem insignificant compared with the increase in RVR with high-dose CsA (Figure 4g). M-COOH caused a 30% decrease in GFR but, surprisingly, caused a decrease in RVR (Figure 4l). This apparent lack of correlation between changes in GFR and RVR may be a reflection of fairly subtle differences in the degree to which the metabolites and CsA act on the afferent and efferent arterioles (as well as glomerular capillaries), or it may indicate that they act via different mechanisms.

Increases in serum potassium with decreased urinary potassium excretion have been reported in patients treated with CsA. There was no evidence of increased potassium reabsorption with any of the metabolites, CsA in Intralipid or methanol, or vehicles alone. However, potassium reabsorption did increase significantly with Sandimmun (CsA, Cremophor, and ethanol). Sodium reabsorption was 99 to 100% at all times and in all experiments. This excludes clearances where urine volume was insufficient (<30 µL/10 min) for sodium analysis, e.g., any clearances where GFR approached zero.

**DISCUSSION**

Despite much study, the mechanisms of CsA nephrotoxicity are still not clearly defined, and it is not known whether the toxic effect is due solely to CsA or to its metabolites and vehicles as well. Several forms of CsA nephrotoxicity have been described; these include: (1) acute, reversible vasoconstriction, which, if severe enough, may result in acute renal failure; (2) proximal tubule pathology, which seems to occur at CsA levels above 1,500 to 2,000 ng/mL, but not at therapeutic levels; (3) renal vascular injury, an inconsistent clinical finding; and (4) chronic, irreversible interstitial fibrosis, which seems to occur only after 6 to 12 months of CsA therapy (1). It is not known whether the chronic vasculointerstitial syndrome is a consequence of prolonged acute effects of CsA. Many nephrologists believe this to be the case, but there is no incontrovertible evidence to support this hypothesis.

The IPK model was chosen to examine several questions about CsA toxicity. The model is valuable because the complications of in vivo studies (systemic toxicity and nonrenal metabolites) can be avoided, and acute functional changes/responses to
drugs can be measured with great accuracy. The model is also well suited to toxicity studies of compounds that are available in extremely limited quantities. The rat kidney may or may not be a good model for acute CsA toxicity in the human kidney, and any extrapolation from one to the other must, of course, be made with caution and only with appropriate confirmation studies in human subjects/human tissue.

Three issues were examined in the studies reported above: (1) the effect of perfusate constituents on the expression of CsA toxicity in the IPK, (2) the effect of CsA vehicles on the IPK, and (3) the effect of CsA metabolites.

Perfusate Constituents

Because of the differences in CsA dose response between investigators using the IPK as a model of acute CsA nephrotoxicity, we postulated that different perfusate constituents may have been responsible for the differences in CsA dose response. There is no evidence from the studies reported above that perfusate constituents [albumin alone, with serum, or with whole blood] had a significant effect on the expression of CsA toxicity in this model. In all three types of perfusate, the effect of CsA, with methanol as vehicle, was modest and comparable; a concentration of 25,000 ng/mL produced a 60% reduction in GFR. We suspect that the CsA vasoconstrictive effect may have been blunted by the use of methanol as the vehicle, because methanol caused significant vasodilation in this model [see CsA Vehicles below].

To our knowledge, there are no reports of the use of fresh whole rat blood in the IPK: washed human red blood cells have been used in previous studies where blood was included in the perfusate [8]. Whole rat blood was used in these studies because renal vascular tone, distal nephron integrity and function, and GFR were closer to normal with blood than without. It seems likely that the IPK is far closer to in vivo conditions with perfusate that contains whole blood. The placement of a transfusion filter in the circuit should have ensured the removal of all cell aggregates and platelets from the perfusate, thus minimizing any contribution that may have had in the effects seen with vehicles, CsA, or metabolites.

The results reported above on three different perfusates—albumin, albumin and serum, or albumin and whole blood—suggest that the discrepancy in CsA dose response between investigators using the IPK is probably not due to differences in perfusate composition.

CsA Vehicles

CsA is extremely hydrophobic and must be dissolved in a lipid or alcohol vehicle in order to be solubilized or emulsified in an aqueous solution. All of the vehicles used to date in this model [Cremophor, ethanol, and methanol] have been found to have effects of their own and, for this reason, were deemed inappropriate vehicles for studies of acute CsA toxicity. Intralipid was chosen as the vehicle in these studies because: (1) it had no apparent effect on the IPK, even at doses 50 times the amount used in these studies; (2) it is a widely used and well-accepted iv agent; and (3) it has the physical properties [fat emulsion] that allow extremely hydrophobic compounds (CsA and metabolites) to be added to an aqueous solution (IPK perfusate). The results reported above indicate that CsA is 25 to 50 times more toxic in this model when the vehicle is Intralipid than when the vehicle is methanol, Cremophor, or Cremophor and ethanol. The reason for this marked enhancement of CsA toxicity with Intralipid vehicle is not known. Intralipid contains two types of particles: (1) small droplets of soybean oil covered [and thus emulsified] by a monolayer of egg yolk phospholipids and (2) monolayer phospholipid micelles containing no core of soybean oil [approximately one third of the phospholipid] [personal communication, Kabivitrum Inc., Alameda, CA]. CsA could potentially be contained in either of these particles: in the oil core of a micelle or in the core of an oil-free micelle. The mechanism for the "Intralipid effect" or its relationship to endothelin, a probable mediator of the CsA effect [19], is unknown, but it is possible that endothelin release from the renal vascular endothelium is enhanced by Intralipid delivery of CsA. We suspect that CsA in a micelle is delivered directly into the endothelial cell either by fusion of the micelle with the cell membrane or via pinocytosis. Whatever the mechanism, it must be something that happens extremely rapidly, because the vasoconstrictive effect of CsA occurs within a few seconds of the CsA reaching the kidney. For this reason, we think that the direct delivery of CsA into the cell via micelle fusion with the cell membrane, with the subsequent immediate release of endothelin, is the most likely mechanism for the rapid and profound vasoconstriction seen in this model. The pharmacokinetics of CsA has been shown to change significantly after the administration of CsA in Intralipid or liposomes, compared with CsA in Sandimmun [20]. The total body clearance and the volume of distribution of CsA were markedly and significantly higher after the iv administration of the liposomal or Intralipid dosage forms of CsA compared with the Sandimmun form. However, the half-life was the same for all three dosage forms [20]. These data support the hypothesis that Intralipid enhances the delivery of CsA to tissues, including the renal vascular endothelium. The enhancement of acute CsA toxicity in the IPK with the Intralipid vehicle is so profound that any further study of this phenomenon
should provide valuable insight into the mechanism(s) of CsA nephrotoxicity. Further studies might include testing antiendothelin antibodies for a blunting of the CsA-Intralipid effect; fractionating the Intralipid mixture to determine which of the particles contain CsA and which fraction has the greatest effect on the IPK; and testing other emulsions or emulsiﬁying systems to see if the "Intralipid effect" occurs with other emulsions or is peculiar to Intralipid.

The results in this model may have profound implications for CsA use in patients with abnormal lipid metabolism (e.g., heart and liver transplant patients) and may ultimately help to explain why some patients are more susceptible to CsA toxicity than others. It is possible that the immunosuppressive effects of CsA, as well as the nephrotoxicity, are enhanced by Intralipid.

CSA Metabolites

To our knowledge, CsA metabolites have never before been studied in the IPK. Purified metabolites are available in very limited quantities, and we were able to test them in the IPK with sufficient numbers of replicates only because the dose response of CsA in this model, with Intralipid as the vehicle, was comparable to that of humans, i.e., a 50% reduction in GFR with 500 ng/mL. There would have been insufficient quantities of the metabolites for study if we had used one of the other vehicles (such as methanol) in which the CsA response was so minimal, i.e., a 50% reduction in GFR at 25,000 ng/mL.

These studies in the IPK model provide the first direct evidence that CsA metabolites have acute nephrotoxic effects comparable to those of the parent drug. These observations may have profound clinical signiﬁcance, because metabolite levels are greatly exceeded CsA levels in patient blood, especially in patients with abnormal liver function (3,5), and may be an important and unrecognized factor in clinical CsA nephrotoxicity. Marked increases in metabolite levels often immediately precede and/or coincide with increases in serum creatinine thought to be due to CsA nephrotoxicity (3; L.M. Shaw, unpublished data). This is intriguing circumstantial evidence for a possible role of metabolites in nephrotoxicity, but to date, there has been no direct evidence that the metabolites contribute to nephrotoxicity. The data reported above provide compelling direct evidence that the metabolites have signiﬁcant acute nephrotoxicity comparable to that of the parent drug in the IPK model. Testing the metabolites for their effects on isolated human glomeruli or afferent/efﬁcient arterioles may be a way to obtain direct evidence for metabolite toxicity in humans. There is a clinical protocol for inhibiting CsA metabolism with the current administration of ketoconazole (21). Other drugs, such as diltiazem, also inhibit CsA metabolism and necessitate the use of markedly reduced doses of CsA (4). Such drugs could be used to test the hypothesis that CsA metabolites contribute to nephrotoxicity. Decreased metabolite levels should result in decreased nephrotoxicity, if the human kidney responds to CsA metabolites as does the rat kidney.

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To the Right Honourable, right worshipful, whether more or less dignified, who have been or hereafter may be my patients, as also to the courteous or discourteous Reader.

Your Honour....... hath often heard it spoken from the mouth of many a well-read and experienced man in Physick that the urine is an Harlot, or a Lyer, and that there is no certain knowledge of any Disease to be gathered from the Urine alone, nor any safe judgement to be exhibited by the same. You have been (likewise) often told, by physicians, that it were far far better for the Physician to see his Patient once, than to view his Urine twenty times.