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A NEW PROPOSAL FOR THE MECHANISM OF CYCLOSPORINE A NEPHROTOXICITY

INHIBITION OF RENAL MICROSOMAL PROTEIN CHAIN ELONGATION FOLLOWING *IN VIVO* CYCLOSPORINE A

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Abstract—In this paper, we report experiments examining the effect of cyclosporine A on "run-off" translation in microsomes isolated from tissues of Sprague–Dawley rats. In microsomes isolated from rat brain, kidney and thymus, cyclosporine A added *in vitro* in concentrations of up to 100 μg/ml did not reduce [^{3}H]L-leucine incorporation relative to controls. A small dose-dependent reduction in [^{3}H]leucine incorporation was observed in microsomes isolated from rat liver when cyclosporine A was added in high concentrations (5 and 6% at 25 and 100 μg/ml). However, when cyclosporine A was injected at 50 mg/kg/day for 10 days, [^{3}H]L-leucine incorporation was inhibited 99.9% in microsomes isolated from kidney. The oral administration of cyclosporine A at 50 mg/kg/day for 6–10 days produced a 75% inhibition of incorporation by isolated renal microsomes. These changes were observed in the absence of measurable reductions in "run-off" transcription measured as [^{3}H]UTP incorporation by renal nuclei exposed to cyclosporine A in concentrations of up to 100 μg/ml *in vitro* or isolated from animals given oral cyclosporine A at 50 mg/kg/day for 6 days. Cross-over experiments were performed using microsomes and microsomal supernatant fractions (cell saps) from tissues of animals treated with cyclosporine A and control vehicle. Renal cell sap from cyclosporine A treated animals inhibited [^{3}H]L-leucine incorporation by microsomes isolated from the kidneys or other tissues of animals treated with control vehicle. These experiments demonstrated that a translation inhibitor was present in the cell sap of cyclosporine A treated animals which could directly block translation elongation in microsomes from control animals. When renal cell sap from both control and cyclosporine A treated animals was added to control microsomes, inhibition was still prominent, suggesting the presence of an inhibitor rather than the absence of an elongation factor. Oral administration of cyclosporine A at 50 mg/kg/day for 6 days depressed renal microsomal [^{3}H]L-leucine incorporation equally in male and female rats to 25% of control. The dose–response relationship for microsomal protein synthesis inhibition after 6 days of oral cyclosporine A administration was: 5 mg/kg, 73.7% of control; 10 mg/kg, 64.1% of control; 25 mg/kg, 54.9% of control and 50 mg/kg, 24.1% of control. Renal microsomal protein synthesis following oral cyclosporine A at 50 mg/kg/day was reduced to 54% of control by day 2 and was maximally inhibited at 25–30% of control by day 4. When cyclosporine A was stopped after 10 days, renal microsomal protein synthesis returned to 59% of control by day 2 and to 99.5% of control by day 4. Oral administration of 50 mg/kg/day cyclosporine A for 10 days was associated with whole blood trough levels of 4.1 μg/ml cyclosporine A and metabolites and with reductions in renal function. Results of our studies are consistent with the hypothesis that a cyclosporine A metabolite, or a product produced in the cell in response to cyclosporine A, directly interferes with translation or the regulation of translation. Translation inhibition may explain the nephrotoxic actions of cyclosporine A.

The fungal cyclic undecapeptide cyclosporine A produces a selective immunotolerance. It inhibits the production by T-helper cells of interleukin-2 and other lymphokines required for cytotoxic T-cell differentiation and proliferation [1, 2]. Cyclosporine A has been proposed to exert inhibitory effects on T-cell activation distal to alloantigen binding, calcium influx, inositol phosphate formation and protein phosphorylation by protein kinase C [3–7]. Cyclosporine A appears to block T-cell activation by inhibiting the transcription of specific messenger RNAs [6–13]. Other cyclic peptides that have been shown to inhibit transcription include the cyclic fungal octapeptide α-amanitin by binding to eukaryotic RNA polymerases [14] and the octadepsipeptide antibiotic triostin by binding to DNA [15].

Cyclosporine A binds avidly to cyclophilin [3, 6], a cytoplasmic protein which has been identified recently as peptidyl-prolyl *cis-trans* isomerase (PPIase; [16, 17]). PPIase is present in the eukaryotic genome in approximately 20 copies and may comprise 0.4% of the protein expressed in the eukaryotic cell [18]. It has been proposed that PPIase folds proteins with prolyl residues as they are synthesized on eukaryotic ribosomes. These observations direct attention to translation rather than transcription as the site of cyclosporine A action and toxicity.

Cyclosporine A produces a dose-dependent toxicity in the kidney, liver, brain and vascular system [1, 2]. T-cells and tissues in which toxicities occur contain high levels of cyclophilin and may accumu-

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late cyclosporine A [4, 6]. Human nephrotoxicity is modeled in Sprague–Dawley and Fischer rats with histological changes in renal tubules, alterations in tubular function and impairment of GFR. These effects require higher doses in rats than in humans [19–21].

In this paper, we report experiments that examined the effects of cyclosporine on protein synthesis using assays of "run-off" translation. In the "run-off" translation assays, [^{3}H]L-leucine was used to measure elongation of protein chains under *in vitro* experimental conditions. These protein chains were initiated under physiological conditions that existed *in vivo* at the time the animal was killed. "Run-off" translation experiments permitted an examination of the effects of cyclosporine A administered *in vivo*, and added *in vitro*, on renal microsomal protein synthesis. Cyclosporine A did not alter "run-off" translation when added *in vitro* to microsomes isolated from various tissues. However, following *in vivo* cyclosporine A administration, "run-off" translation in isolated renal microsomes was inhibited in a dose-dependent, tissue-specific manner in both male and female rats.

MATERIALS AND METHODS

Experimental animals

Sprague–Dawley rats were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN), and maintained under the care of veterinarians. Animals given the control vehicle were pair-fed with animals given cyclosporine A using Wayne's Rodent Diet (Wayne Pet Food Division, Continental Grain Co., Chicago, IL). At sacrifice, animals were cervically stunned and immediately decapitated.

Materials

Cyclosporine A (Sandimmune® I.V. or Sandimmune® Oral Solution) was purchased or received as a gift from the Sandoz Pharmaceutical Corp. (Basel, Switzerland). Cyclosporine A vehicle for oral solutions was obtained from Sandoz or prepared as alcohol (12.5% by volume) dissolved in olive oil. Vehicle for intravenous solutions was obtained from Sandoz or prepared as: alcohol (32.9% by volume) and polyoxyethylated castor oil (Cremophor EL®, a gift from BASF Aktiengesellschaft, West Germany; 650 mg/ml). Reagent grade chemicals used in buffers and translation assays were obtained from the Sigma Chemical Co. (St Louis, MO). Radiochemicals were obtained from ICN Biomedicals (Irvine, CA).

Cyclosporine A administration to rats in vivo *and addition to translation assays* in vitro

Sprague–Dawley rats of either sex (80–250 g) were injected intraperitoneally with cyclosporine A (Sandimmune® I.V.) or vehicle, or given cyclosporine A (Sandimmune® Oral Solution) or vehicle by gastric lavage. When cyclosporine A was added to assays *in vitro*, stocks were made from cyclosporine A powder according to instructions from Dr J. F. Borel of Sandoz Pharmaceuticals. Cyclosporine A (14 mg) was dissolved in 0.5 ml of 96% ethanol and mixed with 0.2 ml of Tween 80. This solution was diluted with water or buffer for addition to assays.

"Run-off" translation assays

Preparation of microsomes. All buffers used in the preparation of microsomes contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor, and 10 μg/ml human placental ribonuclease inhibitor [22]. Tissues (2–10 g) were rinsed (after perfusion in the case of liver) in cold buffer E (0.15 M NaCl, 20 mM HEPES,* pH 7.2), and then minced after standing in 10 ml of cold hypotonic buffer F (10 mM KCl, 15 mM magnesium acetate, 0.7 mM DTT, 10 mM HEPES, pH 7.5). Tissues were homogenized in a Dounce homogenizer with 3 strokes of a loose-fitting glass pestle, followed by 8 strokes of a motor-driven teflon pestle. Two milliliters of buffer G [0.55 M KCl, 2.5 mM spermidine, 0.35 mM DTT, 50% (v/v) glycerol, 0.1 M HEPES, pH 7.5] was added, and the suspension centrifuged at 145,000 *g* for 90 min at 4°.

The top lipoprotein layer was removed by aspiration, the supernatant fraction was retained, and aliquots were used as the source of cofactors in the translation assays. The loose layer remaining above the transparent microsomal pellet was removed by swirling with a small volume of buffer F. The microsomal pellet was gently homogenized in 2 ml of buffer F:G (5:1), and protein levels in microsomal and supernatant fractions were determined using the Bio-Rad® protein assay with crystalline BSA as standard.

Measurement of microsomal [^{3}H]L-*leucine incorporation.* Methods were adapted from those used to measure translation in Ehrlich ascites tumor cells [22]. Microsomes (0.18 to 0.20 mg microsomal protein) were combined in assay tubes with 100 mM KCl, 0.5 mM DTT, 30 mM HEPES (pH 7.5), 1 mM ATP, 0.25 mM GTP, 2 mM magnesium acetate, 0.4 mM spermidine, 5 mM creatine phosphate, 0.18 mg/ml creatine phosphokinase, 50 μM amino acid mix (19 amino acids without leucine, dissolved or suspended in water as a 50 × stock solution), 50 μl microsomal supernatant fraction (cell sap) containing 0.5 mg protein/assay, and 2 μCi [4,5-^{3}H]L-leucine (44 Ci/mmol) in a total volume of 200 μl. Assays were performed for 20 min at 37° in a shaking water bath. Incorporation was quenched by transfer to ice with the addition of 0.2 ml of cold 12% trichloroacetic acid (TCA). Tubes were filled with 6% TCA and centrifuged at 12,000 *g* for 15 min. The supernatant fraction was aspirated off, and the pellet was dissolved in 0.5 ml of cold 0.1 M NaOH. Macromolecular material was reprecipitated with TCA and pelleted, and the pellet was dissolved overnight in capped tubes containing 0.75 ml Soluene®. Samples were counted in 5 ml of scintillation fluid (4 g of diphenyloxazole and 0.1 g of 1,4-bis[2(5-phenyloxazolyl)]-benzene/liter toluene) at an efficiency of 67%.

Microsomal [^{3}H]leucine incorporations increased sharply for 3–4 min, and then increased only slowly through 30 min due to a limited concentration of

* Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; and BSA, bovine serum albumin.

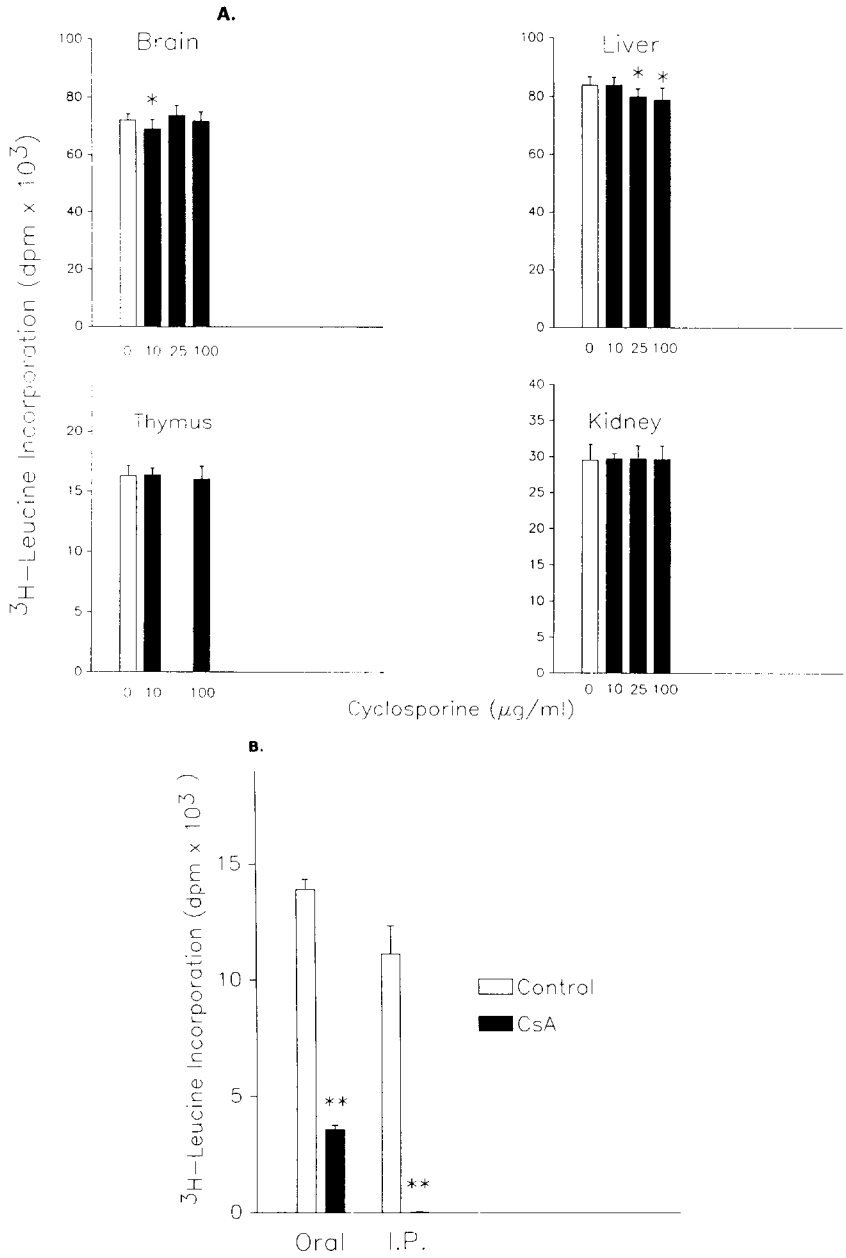

Fig. 1. Microsomal translation after *in vitro* and *in vivo* cyclosporine A. (A) Microsomal translation in the presence of cyclosporine A *in vitro*. Seventeen rats (99.6 ± 11.0 g) of either sex were used to prepare microsomes for "run-off" translation assays (see Materials and Methods). Values are means ± SE for 8 replicates of dpm [^{3}H]L-leucine incorporated/assay (0.20 mg microsomal protein, 0.5 mg cell sap protein) corrected for nonspecific label binding. Addition of vehicle produced no reduction in incorporation as compared to addition of buffer. Statistical significance was determined by Student's *t*-test: brain, * $P < 0.05$; liver, * $P < 0.02$, compared to controls. (B) Microsomal translation after *in vivo* cyclosporine A (CsA). *Intraperitoneal injection*: Ten rats (146.8 ± 16.3 g) of either sex were injected for 10 days with 50 mg/kg/day cyclosporine A (Sandimmune® I.V.) or vehicle. Rats were killed 24 hr after the tenth injection, and microsomes were prepared and assayed for [^{3}H]leucine incorporation as described in Materials and Methods. Values are means ± SE for 8 replicates of dpm [^{3}H]L-leucine incorporated/assay (0.20 mg microsomal protein, 0.5 mg cell sap protein) corrected for nonspecific label binding. Statistical significance was determined by Student's *t*-test: ** $P < 0.001$ for cyclosporine A compared to controls. *Oral administration*. Four female Sprague–Dawley rats (200.8 ± 1.7 g) were given 50 mg/kg/day cyclosporine A (Sandimmune® Oral Solution) or vehicle for 6 days by gastric lavage. Rats were killed 24 hr after the last cyclosporine A administration, and microsomes were prepared and assayed for [^{3}H]leucine incorporation as described in Materials and Methods. Values are means ± SE for 8 replicates of dpm [^{3}H]L-leucine incorporated/assay (0.20 mg microsomal protein, 0.5 mg cell sap protein) corrected for nonspecific label binding. Statistical significance was determined by Student's *t*-test: ** $P < 0.001$ for cyclosporine A compared to controls.

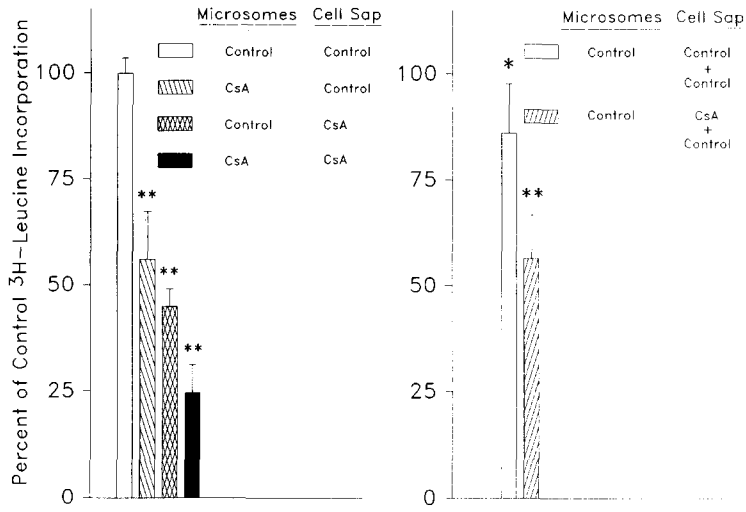

Fig. 2. Effect of cyclosporine A (CsA) on microsomal translation in cross-over experiments using renal microsomes and cell saps. Eighteen female Sprague–Dawley rats (182–212 g) were given 50 mg/kg/day cyclosporine A (Sandimmune® Oral Solution) or vehicle for 6 days by gastric lavage. Rats were killed 24 hr after the last cyclosporine A administration, and microsomes were prepared (0.20 mg microsomal protein, 0.5 mg cell sap protein) and assayed for [^{3}H]leucine incorporation as described in Materials and Methods. Values in the left graph represent 24 replicates each (4 experiments on separate days, each containing 6 replicates), and values in the right graph represent 6 replicates each. Statistical significance was determined by Student's *t*-test: * $P < 0.02$; ** $P < 0.001$ compared to controls.

Table 1. Gender independence for the inhibition of renal microsomal [^{3}H]leucine incorporation produced by *in vivo* cyclosporine A

[^{3}H]Leucine incorporation (% of control)	
Male S/D rats	Female S/D rats
27.5 ± 4.4 (35)	26.0 ± 6.8 (35)

Ten pair-fed male (80–247 g) and ten female (84–199 g) Sprague–Dawley (S/D) rats were treated with 50 mg/kg/day oral cyclosporine A or vehicle for 6 days. Twenty-four hours after the last dose, animals were killed for "run-off" translation assays as described in Materials and Methods. Seven replicate [^{3}H]leucine incorporations were obtained using renal microsomes from each of the 5 male and 5 female experimental animals relative to the 5 male and 5 female control animals. Inhibition is expressed as percent of pair-fed controls to normalize control incorporations. Inhibitory effects were independent of both animal gender and weight. Average control [^{3}H]leucine incorporation was 17,282 dpm for male and 12,804 dpm for female animals. Values were not significantly different as determined by Student's *t*-test.

active initiation factors [22]. [^{3}H]Leucine incorporation was not limited by ATP, GTP, amino acids or the energy-regenerating system. [^{3}H]Leucine incorporations differed between tissues and decreased in individual tissues as animals aged. As negative controls, [^{3}H]leucine incorporation in hepatic microsomes was inhibited 65 and 84% by 2 and 10 μg/ml cycloheximide and in renal microsomes was inhibited 80.8% by 1 mg/ml gentamicin.

RESULTS

We examined the effect of *in vivo* and *in vitro* cyclosporine A on microsomal "run-off" translation measured as [^{3}H]L-leucine incorporation. Microsomes were isolated from the thymus, liver, kidney and brain, and cyclosporine A was added *in vitro* in concentrations up to 100 μg/ml. Cyclosporine A did not reduce [^{3}H]leucine incorporation when added to microsomes from the thymus, kidney or brain. [^{3}H]Leucine incorporation by hepatic microsomes was reduced slightly at the highest cyclosporine A concentrations (25 and 100 μg/ml; Fig. 1A). However, when cyclosporine A was injected intraperitoneally at 50 mg/kg/day for 10 days, a profound inhibition of protein synthesis (99.9%) was observed in microsomes isolated from the kidney 24 hr after the last injection (Fig. 1B). A strong inhibition of protein synthesis (75%) was also observed in renal microsomes isolated 24 hr after 6 or 10 days of oral cyclosporine A at 50 mg/kg (Fig. 1B, see also Table 3).

Renal protein synthesis inhibition was most profound (75%) when microsomes and cell sap were combined from cyclosporine A treated animals (Fig. 2). However, renal cell sap from cyclosporine A treated animals produced an intermediate level of protein synthesis inhibition (55%) when added to control microsomes. A partial inhibition of protein synthesis was also observed when control cell sap was added to microsomes from cyclosporine A treated animals (43%, Fig. 2). When a double aliquot of control cell sap was added to control microsomes, a slight reduction in incorporation (13%) was observed due to assay dilution. When aliquots of cell sap from both cyclosporine A treated and control treated animals were added together to control microsomes, significant inhibition was still apparent (43%, Fig. 2).

When cyclosporine A was given orally for 6 days at 50 mg/kg, renal microsomal protein synthesis inhibition was observed equally in both male and female

Table 2. Dose–response relation for inhibition of renal microsomal [^{3}H]leucine incorporation produced by *in vivo* cyclosporine A

[^{3}H]Leucine incorporation (% of control)			
Oral cyclosporine (mg/kg/day × 6 days)			
5	10	25	50
73.7 ± 9.8 (60) P < 0.05	64.1 ± 9.2 (54) P < 0.001	54.9 ± 11.4 (42) P < 0.001	24.1 ± 3.0 (96) P < 0.001

Pair-fed male and female Sprague–Dawley rats (80–260 g) were given oral cyclosporine A and control vehicle. Twenty-four hours after the last dose, renal microsomes were isolated for "run-off" translation assays described in Materials and Methods. Six replicate [^{3}H]leucine incorporations were obtained using microsomes from each of 7–16 control and experimental animals. [^{3}H]Leucine incorporations are shown as percent of pair-fed controls for normalization. Average control incorporation was 6,378 dpm. Statistical significance was determined by Student's *t*-test compared to controls.

Table 3. Time course for the inhibition of renal microsomal [^{3}H]leucine incorporation produced by *in vivo* cyclosporine A

[^{3}H]Leucine incorporation (% of control)					
Days of oral cyclosporine* (50 mg/kg/day)					
1	2	4	6	8	10
100.8 ± 26.9 (48) (NS)	53.7 ± 11.7 (32) P < 0.001	30.9 ± 5.1 (24) P < 0.001	24.6 ± 1.9 (32) P < 0.001	23.5 ± 8.0 (16) P < 0.001	22.2 ± 2.9 (16) P < 0.001
Days after cessation of cyclosporine A†					
1	2	4		8	
47.1 ± 9.8 (32) P < 0.001	59.3 ± 5.2 (48) P < 0.001	99.5 ± 7.3 (32) NS		98.0 ± 4.7 (32) NS	

* Pair-fed male and female rats (130–201 g) were given oral cyclosporine A (CsA) at 50 mg/kg/day or control vehicle for up to 10 days. Twenty-four hours after the last dose, animals were killed and renal microsomes were isolated for "run-off" translation assays as described in Materials and Methods. Eight replicate [^{3}H]leucine incorporations were obtained using microsomes from each of 2–6 control and experimental animals. Values are shown as percent of pair-fed controls for normalization. Average control incorporation was 12,168 dpm.

† Pair-fed male rats (149–185 g) were given oral CsA at 50 mg/kg/day for 10 days. CsA was stopped and animals were killed for translation assays as previously described. Day 1 animals were killed 24 hr after CsA was stopped. Average control incorporation was 12,136 dpm. Statistical significance was determined by Student's *t*-test compared to controls.

rats at approximately 75% (Table 1). The dose–response relationship for renal microsomal protein synthesis inhibition after the oral administration of cyclosporine A for 6 days was: 5 mg/kg, 73.7% of control; 10 mg/kg, 64.1% of control; 25 mg/kg, 54.9% of control; and 50 mg/kg, 24.1% of control (Table 2). When cyclosporine A was given orally at 50 mg/kg/day, renal microsomal protein synthesis was 53.7% of control on day 2 and was essentially maximally inhibited at 25–30% of control by day 4 (Table 3). Incorporation decreased slowly from 30 to 22% of control through days 4–10 of cyclosporine A. When cyclosporine A was stopped after 10 days, renal microsomal protein synthesis returned to 59% of control by day 2 and 99.5% of control by day 4 (Table 3). Blood levels of cyclosporine A and metabolites were 4.1 μg/ml after 10 days of oral cyclosporine A at 50 mg/kg/day (Table 4), which was associated with significant increases in BUN and reductions in creatinine clearance (Table 5).

DISCUSSION

Our original hypothesis was that cyclosporine A toxicity arises through the inhibition of eukaryotic transcription by RNA polymerases II in a manner similar to that produced by α-amanitin or other small cyclic peptides. We reasoned that toxicity could be tissue specific following the accumulation of cyclosporine A by tissues with high levels of cyclophilin, e.g. kidney cells. However, we were unable to detect changes in "run-off" transcription measured by [^{3}H]UTP incorporation in isolated renal nuclei in the presence of cyclosporine A added in concentrations

Table 4. Whole blood trough cyclosporine A levels in S/D rats

Cyclosporine dose	Blood levels (μg/ml)
50 mg/kg × 10 days (p.o.)	4.1 ± 0.6
25 mg/kg × 10 days (p.o.)	3.2 ± 0.4
25 mg/kg × 10 days (i.p.)	4.1 ± 0.3
12.5 mg/kg × 10 days (p.o.)	2.2 ± 0.4
12.5 mg/kg × 10 days (i.p.)	3.2 ± 0.6

Male Sprague–Dawley (S/D) rats were treated with oral (p.o.) or intraperitoneal (i.p.) cyclosporine A or vehicle for 10 days. Twenty-four hours after the last dose, animals were killed, and blood trough levels of cyclosporine A and metabolites were measured by radioimmunoassay (Sandoz Pharmaceuticals). Values are means ± SE for 6–8 replicates per group.

Table 5. Renal function after cyclosporine A or vehicle

	BUN (mg/dl)	Creatinine clearance (ml/min/100 g)
Vehicle	18 ± 1	0.56 ± 0.04
Oral CsA	28 ± 2*	0.39 ± 0.02*
Intraperitoneal CsA	65 ± 6†	0.23 ± 0.1†

Six pair-fed, male Fischer 344 rats (200–250 g) were given 50 mg/kg/day cyclosporine (CsA) or vehicle by gavage or intraperitoneal injection for 10 days. BUN and creatinine clearance were determined by standard procedures. Values shown are means ± SE for 6 replicates. Statistical significance was determined by Student's *t*-test.

* $P < 0.01$ compared to vehicle.

† $P < 0.01$ compared to oral CsA.

of up to 100 μg/ml or administered orally at 50 mg/kg/day for 6 days. Furthermore, we were unable to detect inhibitory effects of cyclosporine A added to a reconstituted transcription assay using rat thymic nuclear soluble RNA polymerase II and calf thymus DNA as template. This reconstituted transcription assay was inhibited 80% by 2 μg/ml α-amanitin (data not presented).

The fact that transcription inhibition was not observed in renal nuclear transcription assays after *in vivo* or *in vitro* cyclosporine A suggests that, if renal transcription inhibition occurs, it is not extensive. While these experiments suggest that cyclosporine A does not act as a direct or indirect inhibitor of RNA polymerase II activity, they do not rule out the possibility that cyclosporine A may bind to chromatin in a sequence specific manner, or to initiation/elongation factors to antagonize the transcription of specific mRNAs.

Eun *et al.* [23] reported that cyclosporine A inhibited primarily mRNA synthesis, but also DNA and protein synthesis, when added to cultured cells. Inhibition was greatly reduced when transcription and translation were examined *in vitro*, leading the authors to conclude that cyclosporine A was an indirect transcription inhibitor. Suzuki *et al.* [24] reported 50% reductions in rat renal DNA synthesis, 60–70% reductions in RNA synthesis, and 12–20% reductions in protein synthesis over 10–90 days of intraperitoneal cyclosporine A at 2.5 mg/kg/day. Our results are in contrast to these findings. We were unable to demonstrate either a direct or indirect inhibition of transcription in isolated renal nuclei following *in vitro* or *in vivo* cyclosporine A administration.

"Run-off" translation was then examined in microsomes isolated from kidney, thymus, liver and brain. In the presence of cyclosporine A in concentrations of up to 100 μg/ml, protein chain elongation was not inhibited significantly, with the exception of a slight inhibition (4%) in brain microsomes at 10 μg/ml and a small dose-dependent inhibition in hepatic microsomes (5 and 6% at 25 and 100 μg/ml; Fig. 1A). Protein synthesis inhibition in brain microsomes was not observed at cyclosporine A concentrations above 10 μg/ml, suggesting that the inhibition was not dose related. While protein synthesis inhibition in hepatic microsomes was progressive at 25 and 100 μg/ml, the degree of inhibition was small compared to the level of cyclosporine A required to produce the effect. Inhibition seen in hepatic microsomes may be a direct effect of cyclosporine A or due to cyclosporine A metabolites formed during incubation with hepatic microsomes.

Backman *et al.* [25] reported an inhibition of protein synthesis in hepatic microsomes when cyclosporine A was injected *in vivo* or added *in vitro*. Cyclosporine A administration partially reduced the phenobarbital-mediated induction of hepatic cytochrome P-450 enzymes [25], consistent with an inhibition of protein synthesis. In our studies, hepatic microsomal protein synthesis was reduced only by *in vitro* cyclosporine A concentrations of 25–100 μg/ml (Fig. 1A). We conclude from our studies that cyclosporine A has a tissue-specific, but minor, direct inhibitory effect on hepatic microsomal protein synthesis.

While direct addition of cyclosporine A produced minor microsomal translation inhibition (Fig. 1A), the injection of 50 mg/kg cyclosporine A for 10 days produced a profound inhibition of protein synthesis (99.9%) in subsequently isolated renal microsomes (Fig. 1B). When 50 mg/kg/day cyclosporine A was administered for 6–10 days by the oral route, it also produced a dramatic reduction in renal microsomal protein synthesis (75%, Fig. 1B). This finding is consistent with the reported 35% bioavailability of oral cyclosporine [1] and our observation that intraperitoneal injection of cyclosporine A for 10 days in the Sprague–Dawley rat produced the same trough blood levels as the oral administration of twice the amount of cyclosporine A (Table 4). Microsomal protein synthesis in liver, brain and heart was not reduced significantly following *in vivo* cyclosporine A (data not shown). As cyclosporine A added directly to microsomes produced no reduction in protein synthesis, these results suggest that cyclosporine indirectly produces a tissue-specific inhibition of translation.

"Run-off" translation assays use purified microsomes, substrates, an energy-generating system, amino acids, and the microsomal supernatant fraction (cell sap) as a source of translation cofactors. Cross-over experiments using the microsomal and cell sap fractions from cyclosporine and control

treated animals provided further information on translation inhibition. Protein synthesis inhibition was most profound (75%) when renal microsomes and cell sap were combined from cyclosporine A treated animals (Fig. 2). Renal cell sap from cyclosporine A treated animals produced a partial inhibition of protein synthesis (55%) when added to renal control microsomes. A reduced degree of inhibition (43%) was observed when renal control cell sap was added to renal microsomes from cyclosporine A treated animals (Fig. 2).

These data demonstrate that the translation inhibitor is present in both the cell sap and microsomal fractions of renal tissue, and that the inhibitor is diffusible. The inhibitor directly blocks elongation, as is demonstrated by the experiment in which renal cell sap from cyclosporine A treated animals inhibited renal microsomes from vehicle treated animals (Fig. 2). The pronounced inhibition observed in these cross-over experiments, in which 50 μl of renal cell sap from cyclosporine A treated animals was added to a 200 μl assay volume containing control renal microsomes, suggests that inhibition of translation elongation rather than translation initiation can explain the observed inhibitory effects. The pronounced inhibition observed under these experimental conditions also strongly suggests that the primary site of renal protein synthesis inhibition is at the level of translation rather than at the level of transcription.

The addition of double aliquots of control cell sap to control microsomes resulted in a slight reduction in incorporation (13%) due to assay dilution (Fig. 2). However, when cell saps from control and cyclosporine treated animals were both added to control microsomes, inhibition was still observed (43%, Fig. 2). This experiment suggests that inhibition is due to the presence of an inhibitor rather than to the absence of an elongation factor.

Further observations consistent with the hypothesis that cyclosporine A administration inhibits protein synthesis are the following. After unilateral nephrectomy in rats, cyclosporine A delays or prevents compensatory hypertrophy of the remaining kidney [26]. Cyclosporine A also antagonizes renal compensatory regeneration following ischemia [27]. Cyclosporine A reduces renal mitochondrial oxidative phosphorylation, an effect that appears to be associated with both a direct inhibition of the mitochondrial electron transport chain and reduced levels of cytochrome oxidase and F_1-ATPase activity [28–31]. Since subunits of these mitochondrial enzymes are synthesized on cytoplasmic ribosomes, inhibition of cytoplasmic protein synthesis would be expected to produce deficits in mitochondrial enzymes and mitochondrial function [32, 33]. The capacity of cyclosporine A to reduce mitochondrial enzyme levels supports our observation of translation inhibition, and further suggests a primary role for translation inhibition in drug toxicity.

Renal microsomal protein synthesis inhibition was observed equally in both male and female rats (Table 1). Table 2 demonstrates that the inhibition of renal microsomal protein synthesis is dose related. The dose–response relationship for renal microsomal protein synthesis inhibition demonstrates that inhibition is significant at doses used clinically. At oral doses of 5 and 10 mg/kg/day for 6 days, renal microsomal protein synthesis was reduced to 73.7 and 64.1% of control respectively. Renal microsomal protein synthesis was reduced to 54% of control by day 2 of oral cyclosporine A administration at 50 mg/kg/day and was maximally inhibited by day 4 at 25–30% of control (Table 3). When cyclosporine A was withdrawn, protein synthesis returned to 59% of control by day 2 and recovery was complete by day 4. With a half-life of 16–24 hr, cyclosporine A would essentially reach plateau blood levels in 4 half-lives or by day 4, and with cessation would also be essentially completely eliminated by day 4. The degree of inhibition of renal microsomal protein synthesis appears to directly follow the first order approach of cyclosporine A to equilibrium levels in the blood. As cyclosporine A has been shown to be rapidly metabolized, inhibition may also be associated with the build-up of a cyclosporine A metabolite.

Intraperitoneal injection of cyclosporine A for 10 days was associated with blood trough levels of cyclosporine A and metabolites that were the same as those produced by the oral administration of twice the dose (Table 4). As expected, intraperitoneal injection of 50 mg/kg/day cyclosporine A for 10 days produced more renal impairment measured as increases in BUN and decreases in creatinine clearance than did oral administration (Table 5). Oral administration of 50 mg/kg/day for 10 days produced blood trough levels which were approximately 8-fold those that begin to produce toxic effects in humans (Table 4); however, rats have been reported to be more resistant than humans to cyclosporine A nephrotoxicity [19–21].

Cyclosporine A induced translation inhibition in the kidney required *in vivo* cyclosporine administration. This observation is the basis of our hypothesis that a cyclosporine metabolite, or a product induced or produced in the renal cell in response to cyclosporine A, directly interferes with translation or the regulation of translation. If protein synthesis inhibition is produced by a metabolite of cyclosporine, the metabolite may be formed specifically in the kidney or produced in the liver and concentrated in the kidney. There are several reports suggesting that hepatic cytochrome P-450 enzyme induction by phenobarbital decreases cyclosporine A nephrotoxicity [34–37]. These findings do not rule out the formation of a toxic metabolite, because induction of a specific cytochrome P-450 pathway by phenobarbital could increase the metabolism of cyclosporine A to a nontoxic derivative, reducing the metabolism of cyclosporine A to a toxic metabolite by a minor noninduced pathway. This may particularly be the case for a drug like cyclosporine A in which many primary and secondary metabolites are formed. Furthermore, a toxic metabolite or secondary metabolite may be formed in renal cells, and an elevation of hepatic metabolism may limit the amount of cyclosporine A or metabolite that can be formed in or accumulated by the kidney.

Cyclosporine A has been proposed to reduce the transcription of specific mRNAs [6–13]. These could include those encoding gene products involved in

translation or in the regulation of translation. Alternatively, the primary site of cyclosporine A inhibition may be translational, leading to secondary, specific reductions in transcription due to decreases in the synthesis of factors involved in transcription initiation or elongation. Translation inhibition following *in vivo* cyclosporine A may be due to a second messenger or a gene product produced in the kidney, or at other sites in the body and taken up by the kidney.

We propose that renal microsomal protein synthesis inhibition directly accounts for, or is strongly contributory to, cyclosporine A induced nephrotoxicity. The identification of cyclophilin as PPIase, and its proposed role in folding proteins during ribosomal synthesis [16, 17], suggest that microsomal PPIase could be the site of protein synthesis inhibition observed in our studies. We are conducting experiments to test this possibility.

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