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DISSOCIATION OF DECREASES IN RENAL CELLULAR ENERGETICS AND RECOVERY OF RENAL MICROSOMAL TRANSLATION DURING CHRONIC CYCLOSPORINE A ADMINISTRATION

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Abstract—Male Sprague–Dawley rats were given oral cyclosporine A or control vehicle, and renal protein synthesis and renal ATP levels were examined. Acute oral cyclosporine A at 5, 10, 25 and 50 mg/kg/day for 6 days reduced [^{3}H]L-leucine incorporation by isolated renal microsomes to 76.2, 56.8, 44.3 and 29.5% of control incorporations, respectively. No significant changes in renal ATP levels were detected by NMR spectroscopy after acute oral cyclosporine A administration at the doses indicated. However, during chronic exposure to cyclosporine A at doses of 5 and 25 mg/kg/day for 30, 60 and 90 days, there was a recovery of renal microsomal protein synthesis by day 30 at 5 mg/kg/day, and by day 45 at 25 mg/kg/day. NMR spectroscopy of the kidneys of these rats demonstrated decreases in renal ATP level by day 60 in animals given cyclosporine A at 25 mg/kg/day. Cyclosporine A administration produced a renal acidosis and up to a 40% decrease in renal ATP level by day 90 in rats fed cyclosporine A at 25 mg/kg. No apparent histologic abnormalities were observed in the ATP-deficient renal tissue by NMR imaging. Reductions in renal ATP level suggest that the recovery of renal microsomal protein synthesis is aberrant in the continued presence of cyclosporine A, or that mitochondria are direct sites of cyclosporine A toxicity.

The dose-limiting side-effect of cyclosporine A is commonly nephrotoxicity [1–3]. Our work demonstrates that acute *in vivo* cyclosporine A produces dose-dependent, tissue-specific reductions in renal protein synthesis measured as [^{3}H]L-leucine incorporation in subsequently isolated renal microsomes [4, 5]. Cyclosporine A added *in vitro* does not reduce incorporation by renal microsomes. At high cyclosporine A doses, a complete inhibition of microsomal [^{3}H]leucine incorporation is observed in the absence of measurable reductions in [^{3}H]UTP incorporation in renal nuclear "run-off" transcription assays [4, 5, §].

In recent experiments, we have examined the effects of acute and chronic cyclosporine A administration on renal protein synthesis and renal ATP levels using NMR spectroscopy. In this paper, we provide preliminary evidence that NMR spectroscopy can detect changes in pH and ATP level in rat kidneys during the administration of cyclosporine A in doses that have been shown to reduce renal function. Early, dramatic reductions in rat renal microsomal protein synthesis were observed in the absence of changes in renal pH and ATP levels. A time- and dose-dependent recovery of renal microsomal protein synthesis was then observed during continued cyclosporine A administration. However, as renal protein synthesis returned to control values, reductions in the level of ATP were observed in NMR spectra of the kidneys of these animals. Reductions in renal ATP level suggest that the recovery of microsomal protein synthesis is aberrant in the continued presence of cyclosporine A, or that mitochondria are direct sites of toxicity during chronic cyclosporine A administration.

MATERIALS AND METHODS

Experimental animals

Sprague–Dawley rats were obtained from Harlan Sprague–Dawley, Inc., Indianapolis, IN, and maintained under the care of veterinarians. Cyclosporine A or control vehicle was given by gavage in volumes of 0.1 to 0.2 mL. Rats given control vehicle were pair-fed with Wayne's Rodent Diet (Wayne Pet Food Division, Continental Grain Co., Chicago, IL) to animals given cyclosporine A. Cyclosporine A or control vehicle was administered to rats by gavage for 6, 15–17, 30, 44–45, 61–63 and 89–91 days. Rats given oral cyclosporine A or vehicle for 6 days were used either for NMR spectra or for the preparation of renal microsomes. At all other times, rats were anesthetized as described below for NMR spectra or images and killed 1–2 days later for the preparation of renal microsomes. Animals were killed by decapitation 24 hr after the last drug or vehicle dose.

Materials

Cyclosporine A (Sandimmune^R Oral Solution) and cyclosporine A oral solution vehicle were purchased or received as a gift from the Sandoz

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Pharmaceutical Corp., Basel, Switzerland. Reagent grade chemicals used in buffers and translation assays were obtained from the Sigma Chemical Co., St. Louis, MO, and radiochemicals from ICN Biomedicals, Irvine, CA.

"Run-off" translation elongation assays

Preparation of microsomes and measurement of microsomal "run-off" [^{3}H]L-leucine incorporation. Microsomes were prepared for "run-off" translation assays as previously described [5]. All buffers used in the preparation of microsomes contained 0.1 mmol phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor and 10 μg/mL human placental ribonuclease inhibitor. Protein levels in microsomal and supernatant fractions were determined using the Bio-Rad^R protein assay. Methods for assaying the elongation phase of translation in microsomes were adapted from those used to measure translation elongation in Ehrlich ascites tumor cells [6]. [^{3}H]Leucine incorporation in renal microsomes was inhibited 70 and 90% by 2 and 10 μg/mL cycloheximide and, as expected, incorporation was not reduced by 2 μg/mL of the eukaryotic translation initiation inhibitor aurintricarboxylic acid.

Renal microsomes (0.18 to 0.20 mg microsomal protein) were combined in assay tubes with 100 mmol KCl, 2 mmol magnesium acetate, 0.5 mmol dithiothreitol (DTT), 1 mmol ATP (pH 7.0), 0.25 mmol GTP (pH 7.0), 0.4 mmol spermidine, 5 mmol 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 50X stock solution), 50 μL renal microsomal supernatant (cell sap) containing 0.5 mg protein/assay, 2 μCi [4,5-^{3}H]L-leucine (44 Ci/mmol) and 30 mmol HEPES (pH 7.5) in a total volume of 200 μL. Assays were incubated 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 was aspirated off and the pellet dissolved in 0.5 mL of cold 0.1 M NaOH. Macromolecular material was reprecipitated with TCA and then centrifuged, and the pellet was dissolved overnight in capped tubes containing 0.75 mL of solubilizer (Soluene^R). Samples were counted at an efficiency of 67% in a Beckman 1801 Scintillation Spectrometer in 5 mL scintillation fluid consisting of 4 g diphenyloxazole and 0.1 g 1,4-bis[2(5-phenyloxazolyl)]benzene/liter toluene.

Microsomal [^{3}H]leucine incorporations increased linearly for 1.5–2.0 min, continued to increase through 3–5 min, and then increased only very slightly through 30 min. [^{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.

Nuclear magnetic resonance spectroscopy and imaging

Spectroscopy. All rats were anesthetized with an intraperitoneal injection of 150 mg/kg sodium 5-ethyl-5-(1' - methylpropyl)-2-thiobarbiturate (Inactin^R). The right kidney was exposed following an incision through the paraspinus muscle and the right renal artery was tied off. The left kidney was exposed and held elevated by two sutures through the surrounding muscle. A two-turn transmission line surface coil was secured over the left kidney [7]. Experiments were performed at 4.7 Tesla using a 33 cm bore General Electric Medical Systems CSI unit. The NMR coil was tuned to the frequency of protons and the magnetic field homogeneity was optimized by maximizing the signal from water. The coil was then tuned to the frequency of phosphorus, and a total of 128 scans were summed and treated with 20 Hz of exponential broadening. The repetition time was 10.2 sec. The chemical shift of phosphocreatine was set to 0 ppm when observed in the spectra. Alternatively, the resonance from the α-phosphate of ATP was assigned a shift of −7.7 ppm. A representative NMR spectra is shown in Fig. 1. Renal ATP/ADP + ATP ratios shown in Table 3, and renal pH values discussed in the text, were calculated as described in the legend to Fig. 1 [8–10].

Imaging. Matched pairs of experimental and control rats were imaged at 2-week intervals during treatment. Rats were positioned in a 2 in. × 6 in. × 6 in. plexiglass container with inflow and outflow tubes. Anesthesia was induced with a 100 cc/min flow of 2% halothane. A 10 mm centrifuge tube containing a 0.1 N solution of copper sulfate was positioned between rats as an internal intensity standard. The entire box was positioned in the extremity coil of a 1.5 Tesla General Electric Medical Systems Signa unit. Sets of T1-weighted axial images were obtained with a repetition time of 600 msec and an echo time of 20 msec. The slice thickness was 3 mm, the field of view was 8 cm, and the resolution was 350 μM. T2-weighted coronal images were acquired with a repetition time of 2.5 sec and an echo time of 80 msec. Renal signal intensity was measured in three regions of interest and compared to the intensity standard for each rat.

RESULTS

We have shown that *in vivo* cyclosporine A results in a tissue-specific reduction in renal microsomal protein synthesis that is independent of animal age and gender [5]. We have also demonstrated previously that 4 days of oral cyclosporine A administration are sufficient to produce maximal renal microsomal inhibition, and that there are concomitant increases in BUN and decreases in creatinine clearance by day 10 of cyclosporine A administration [5]. Recent experiments suggest an elevation of BUN and serum creatinine by day 1 of oral cyclosporine A at 50 mg/kg and by day 6 at 10 mg/kg.*

When cyclosporine A was administered orally for 6 days, it produced a dose-dependent reduction in renal microsomal [^{3}H]L-leucine incorporation, as shown in Table 1. These changes were not associated with reductions in renal pH or ATP levels determined by NMR spectra (data not shown). With chronic

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Table 1. Inhibition of protein synthesis in microsomes isolated from the kidneys of rats given oral cyclosporine A (CsA) for 6 days

Oral dose of CsA (mg/kg/day)	[^{3}H]L-Leucine incorporated (dpm/assay)		
	Control	CsA	% of Control
5	6866 ± 198 (17)	5234 ± 173 (18)	76.2 ($P < 0.001$)
10	6492 ± 185 (18)	3685 ± 591 (18)	56.8 ($P < 0.001$)
25	7325 ± 202 (12)	3246 ± 808 (12)	44.3 ($P < 0.001$)
50	7134 ± 187 (8)	2103 ± 93 (8)	29.5 ($P < 0.001$)

Male rats (164–198 g initial weights) were given oral cyclosporine A at the doses indicated or pair-fed and given control vehicle. Animals were used for NMR spectroscopy or for the preparation of renal microsomes. Each renal microsomal assay contained 0.25 mg microsomal protein and 0.5 mg renal cytoplasmic protein. [^{3}H]L-Leucine incorporations were corrected for nonspecific label binding (see Materials and Methods). Values are means ± SEM; the numbers in parentheses refer to the number of assays performed.

Table 2. Inhibition of protein synthesis in microsomes isolated from the kidneys of rats given oral cyclosporine A for 15–91 days

Length of treatment	[^{3}H]L-Leucine incorporated (dpm/assay)		
	Controls	Cyclosporine A	
		5 mg/kg	25 mg/kg
15–17 days (5)	15,011 ± 382 (39) 100.0%	12,062 ± 332 (40) 80.4% ($P < 0.001$)	12,149 ± 355 (38) 80.9% ($P < 0.001$)
30 days (2)	12,899 ± 293 (16) 100.0%	13,639 ± 915 (16) 105.7% (NS)	10,941 ± 663 (16) 84.8% ($P < 0.02$)
44–45 days (2)	10,345 ± 237 (16) 100.0%	13,614 ± 198 (16) 131.6% ($P < 0.001$)	9522 ± 1074 (16) 92.0% (NS)
61–63 days (5)	10,423 ± 663 (38) 100.0%	12,407 ± 923 (38) 119.0% (NS)	9138 ± 536 (38) 87.7% (NS)
89–91 days (2)	8307 ± 74 (16) 100.0%	10,537 ± 611 (16) 126.8% ($P < 0.01$)	8571 ± 1022 (16) 102.8% (NS)

Male rats (164–198 g initial weights) were given oral cyclosporine A at the doses indicated or pair-fed and given control vehicle. Animals were anesthetized with halothane for NMR spectroscopy and killed 1–2 days later for the preparation of renal microsomes. Halothane alone did not reduce renal microsomal incorporation. Each renal microsomal assay contained 0.25 mg microsomal protein and 0.5 mg renal cytoplasmic protein. [^{3}H]L-Leucine incorporations were corrected for nonspecific label binding (see Materials and Methods). Values are means ± SEM. Numbers in parentheses next to days refers to the number of individual animals used in the experiments, while numbers under incorporations refer to the number of assays performed.

cyclosporine A administration, however, renal microsomal [^{3}H]leucine incorporation returned to control values in a dose- and time-dependent fashion. As shown in Table 2, oral administration of 5 or 25 mg/kg/day cyclosporine A for 15–17 days resulted in renal [^{3}H]L-leucine incorporations that were 80.4 and 80.9% of control values, respectively. By 30 days of continuous cyclosporine A administration,

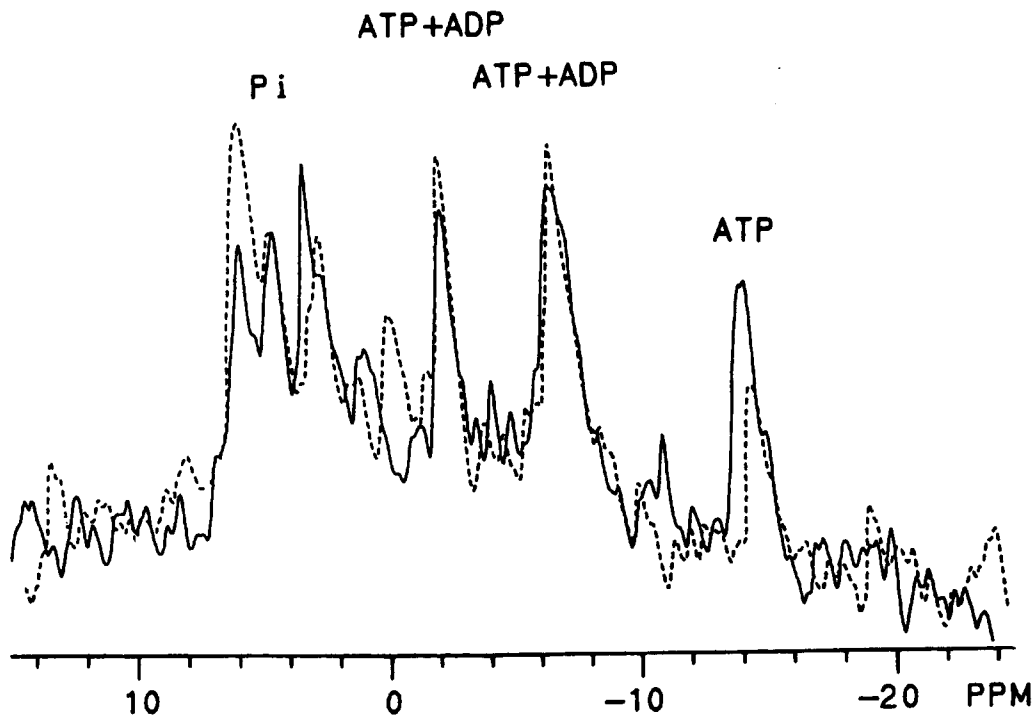

Fig. 1. NMR spectra of rat kidneys after 90 days of oral cyclosporine A or control vehicle. Rats were given an oral control vehicle (solid line) or oral cyclosporine A at 25 mg/kg/day (dashed line) for 90 days. The ATP/ADP + ATP ratios shown in Table 3 were calculated as the ratio of the areas of the β-ATP peak at −15.8 ppm divided by the area of the γ-ATP + β-ADP peak at −2.2 ppm [8, 9]. Renal pH was estimated from the difference in chemical shift between the signal from inorganic phosphate and the peak for α-ATP at −7.7 ppm according to the formula of Shine *et al.* [10].

renal microsomal [^{3}H]leucine incorporation returned to control values in the rats given 5 mg/kg/day cyclosporine A and to 84.8% of control values in rats given 25 mg/kg/day. Renal microsomal incorporation in animals given 25 mg/kg/day cyclosporine A returned to control values by 44–45 days, by which time the animals which continued on 5 mg/kg/day cyclosporine A had exceeded control levels of renal protein synthesis (Table 2).

NMR spectra from kidneys of rats given acute and chronic cyclosporine A and control vehicle were used to determine renal intracellular pH and ATP/ADP + ATP ratios as indicated in the legend to Fig. 1. When animals were given oral control vehicle or cyclosporine A at 50 mg/kg/day for 6 days, renal NMR spectra from control and cyclosporine A-treated animals were superimposable, indicating an absence of significant changes in renal pHs or ATP levels (see legend to Fig. 1). Reductions in renal ATP levels determined by NMR spectroscopy in animals given chronic oral cyclosporine A are shown in Table 3. The decline in ATP level was dose and time dependent. A comparison of Table 2 with Table 3 demonstrates that, although renal protein synthesis had recovered by day 60, renal ATP level had declined. Chronic administration of 5 mg/kg cyclosporine A resulted in a decline of the ratio of ATP/ADP + ATP to 97.8% of control by day 30, to 79.8% of control by day 60, and to 74.7% of control by day 90 (average of three control and two experimental animals at each time period). Renal

Table 3. Renal ATP levels following chronic cyclosporine A

Days	ATP / ADP + ATP		
	Control vehicle	Cyclosporine A	
		5 mg/kg/day	25 mg/kg/day
30	0.91	0.89	0.88
	(3)	(2)	(2)
	100.0%	97.8%	96.7%
60	0.89	0.71	0.57
	(3)	(2)	(2)
	100.0%	79.8%	64.0%
90	0.83	0.62	0.50
	(3)	(2)	(2)
	100.0%	74.7%	60.2%

Values are means of NMR spectra obtained, as described in Materials and Methods, from three control and two experimental animals. ATP/ADP + ATP ratios were determined by NMR spectroscopy as described in the legend to Fig. 1.

pH values fell from 7.3 ± 0.1 (N = 10) in control animals given cyclosporine A vehicle for 90 days to 7.2 ± 0.1 (N = 5; NS) in animals given cyclosporine A at 5 mg/kg/day for 90 days. When cyclosporine A was given at 25 mg/kg/day, the ratio of ATP/ADP + ATP declined to 96.7% of control by day 30, to 64.0% of control by day 60, and to 60.2% of control by day 90 (average of three control and two experimental animals at each time period). As shown in Table 3, the trend in ATP production was clearly a function of the dose and duration of cyclosporine A. Renal pH fell from 7.3 ± 0.1 (N = 10) in controls given vehicle for 90 days to 7.0 ± 0.1 (N = 5; $P < 0.05$) in animals given cyclosporine A at 25 mg/kg/day for 90 days. NMR images of the kidneys of rats given chronic cyclosporine A at 5 and 25 mg/kg/day for up to 90 days showed no apparent changes compared to kidneys of control animals (images not shown).

DISCUSSION

Cyclosporine A may produce dose-dependent toxicities in a variety of tissues, including the vascular system, liver, CNS, and most prominently, the kidney [1–3]. Human nephrotoxicity can be modeled in rats at doses somewhat higher than those that produce nephrotoxicity in humans [1]. Oral cyclosporine A at 5–20 mg/kg/day in humans is accompanied by nephrotoxicity seen as a chronic impairment of renal blood flow and glomerular filtration, leading to elevations in plasma creatinine and BUN. There are dose-dependent alterations in tubular function that are variably associated with renal histologic changes, in extreme cases taking the form of glomerulosclerosis, interstitial fibrosis and end stage renal disease [1–3]. These same effects are observed in rats at doses greater than 20 mg/kg/day and are pronounced at 40–100 mg/kg/day [11].

Published reports suggest that reductions in renal function measured as elevated BUN and serum creatinine are cyclosporine dose and time dependent. Cyclosporine A at doses of 25 mg/kg/day produces only mild renal impairment by day 14 [12]. In unilaterally nephrectomized rats subjected to ischemia, 40 mg/kg/day cyclosporine A lowers renal function by day 9, while 20 mg/kg/day does not [13]. BUN levels are elevated by day 3, and creatinine levels by day 7, when cyclosporine A is given orally at 50 mg/kg/day [14]. We found that cyclosporine A in oral and intraperitoneal doses of 50 mg/kg/day produces significant reductions in creatinine clearance and elevations in BUN by day 10 [5], and in recent experiments as early as day 1.

While effects on eicosanoids may explain the acute effects of cyclosporin A on renal function [14–16], they are unlikely to provide an explanation for the dose-dependent inhibition of renal protein synthesis and decreases in renal ATP level reported in this paper. Cyclosporine A-induced reductions in renal [^{3}H]leucine incorporation begin by day 2 and are fully developed by days 4–6 of cyclosporine A administration [5]. As determined in the present study, renal ATP levels and renal pH values were not significantly different from controls at days 4–6 (data not shown). Maximal reductions in renal microsomal protein synthesis persisted at least through days 6–8 of cyclosporine A. By day 10 of continued cyclosporine A administration, however, there was variable evidence of the recovery of renal protein synthesis that became distinct by day 15. Renal microsomal protein synthesis returned to control values by day 30 with oral administration of 5 mg/kg/day cyclosporine A, and by day 45 with 25 mg/kg/day cyclosporine A (Table 2). Thus, elevations in BUN and serum creatinine levels and reductions in renal microsomal protein synthesis can be dissociated.

Although renal microsomal protein synthesis returned to control values with chronic cyclosporine A administration, renal ATP levels fell. Reductions in renal ATP could be due to a reduction in ATP synthesis or to an increase in ATP turnover. Published reports have suggested that mitochondria are sites of cyclosporine A toxicity [17–19]. Ferrero and Marni [18] reported that intraperitoneal injection of 5–15 mg/kg/day cyclosporine A for 15 days sharply reduces ATP levels and increases cAMP levels in lymphocytes. Hay *et al.* [19] reported that, following cyclosporine A, there are reductions in subunits IV and V of cytochrome oxidase and the β-subunit of F_1-ATPase in renal mitochondria from spontaneously hypertensive rats. Since these mitochondrial enzyme subunits are synthesized on cytoplasmic ribosomes, the authors suggested that mitochondrial defects could be due to an inhibition of mitochondrial biogenesis due to impaired cytoplasmic protein synthesis.

In this paper we have demonstrated that reductions in renal microsomal [^{3}H]leucine incorporation and reductions in ATP level are not temporally related (Tables 1–3). Renal microsomal [^{3}H]leucine incorporation is increasingly inhibited in a dose-dependent fashion over 4 days of oral cyclosporine A administration at 5–50 mg/kg/day (Table 1; [5]) and returns to control values in 4 days after cyclosporine A is withdrawn [5]. When cyclosporine A was given continuously, there was a return to control values by 30–45 days as a function of chronic dose (5 and 25 mg/kg/day; Tables 2 and 3). During the recovery of renal microsomal protein synthesis, renal ATP levels declined as a function of time and cyclosporine A dose (Fig. 1 and Table 3).

In addition to reductions in ATP levels, renal acidosis was also observed by NMR spectroscopy. The reduction in pH must be confined to renal tissues, since animals given oral cyclosporine A at doses of up to 50 mg/kg/day appeared normal and consumed the same amount of food as control animals given vehicle (data not presented). A renal pH of 7.0 ± 0.1 in animals given oral cyclosporine A at 25 mg/kg/day for 90 days, compared to a renal pH of 7.3 ± 0.1 in control animals, indicates a disturbance in renal acid–base balance. Reductions in ATP with concomitant observed disturbances in glycerol phosphocholine and glycerol phosphoethanolamine levels (data not shown) suggest a high level of anaerobic metabolism or mitochondrial damage [19].

Cyclosporine A has been shown to bind to the cytoplasmic receptor cyclophilin [20], which is

identical to peptidyl-prolyl *cis-trans* isomerase (PPIase; [21, 22]). The immunomodulatory compound FK506 binds to a cytoplasmic receptor separate from cyclophilin, but which also has PPIase activity [23]. PPIase has been proposed to be a chaperone protein, responsible for folding proteins as they are synthesized. Inhibition of a PPIase activity associated with ribosomal polypeptide chain elongation could putatively account for the inhibition of microsomal translation elongation observed in our experiments following *in vivo* cyclosporine A.

Translation elongation is not totally inhibited in kidney until very high doses of cyclosporine A are administered [4, 5]. Residual protein synthesis in the face of high cyclosporine A doses, or continuing protein synthesis in the presence of lower cyclosporine A doses, could permit adaptive cellular responses involving translation. With chronic cyclosporine A administration, adaptive cellular products could be produced which allow or increase ribosomal elongation at the expense of translation accuracy. The adaptive components could be part of an SOS, heat or chemical shock response affecting translation. Under these conditions, ATP pools might be depleted by both the ribosomal synthesis of faulty mitochondrial proteins and their continued degradation after tagging with ubiquitin. Nephrotoxicity may be caused by the tissue-specific inhibition of translation elongation, or be associated with the adaptive response involving return of translation to control levels with concomitant decreases in renal ATP.

In renal transplant patients, it may be difficult to differentiate between post-transplant nephrotoxicity produced by organ rejection and that produced by cyclosporine A. The erratic absorption of oral cyclosporine A complicates the problem of achieving appropriate immunomodulatory concentrations (100–400 ng/mL) without producing nephrotoxicity. The clinical resolution of this question may require renal biopsy. The data presented in this paper provide preliminary evidence that nuclear magnetic resonance spectra may be used to assess cyclosporine A nephrotoxicity by examining renal ATP level. NMR spectroscopy may provide data obtained non-invasively, allowing an assessment of graft progression and adjustments of the cyclosporine A treatment schedule.

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