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DIFFERENTIAL EFFECTS OF CYCLOSPORIN ON HEPATIC AND RENAL HEME, CYTOCHROME P-450 AND DRUG METABOLISM

POSSIBLE ROLE IN NEPHROTOXICITY OF THE DRUG

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Abstract—Treatment of rats with 25 or 50 mg/kg cyclosporin A for 6 days elicited vastly different responses in hepatic and renal heme and drug metabolism activities. In the liver, cytochrome P-450 concentration was decreased significantly (to 70–75% of the control). This was accompanied by a marked reduction in benzo[*a*]pyrene hydroxylase activity (to 20–28% of the control). Aniline hydroxylation was also decreased, but to a lesser extent (to 77% of the control). In contrast, in the kidney cytochrome P-450 concentration was significantly increased to (145–170% of the control), along with a modest decrease in benzo[*a*]pyrene hydroxylation activity. In this organ, the concentration of porphyrins was severely decreased (to 30% of the control). Also, the activities of δ-aminolevulinate (ALA) synthetase and ALA dehydratase, as well as that of heme oxygenase, were inhibited. It is suggested that in the kidney the inhibition of degradation, rather than an enhanced rate of synthesis of the heme molecule, contributes to the observed increase in cytochrome P-450 concentration. In the liver, the decrease in the cytochrome concentration could not be explained in terms of an alteration in the rate of heme biosynthesis or degradation. Therefore, the observed decrease in cytochrome P-450 concentration could reflect the direct inactivation of the hemoprotein or regulation of apoprotein production by cyclosporin and/or its metabolites(s). The possible relevance of the observations to cyclosporin nephrotoxicity is discussed.

Cyclosporin is an immunosuppressive drug widely used in transplant protocols. Its therapeutic action is selective for the suppression of lymphocytes which mediate graft rejection [1]. Cyclosporin, however, is nephrotoxic, and this toxicity constitutes the main dose-limiting factor in the utilization of the drug [1, 2].

Although substantial progress has been made in defining the spectrum of biological activities of cyclosporin, for the most part, the mechanism(s) of nephrotoxicity of the drug has remained elusive. A current leading theory postulates an altered renal hemodynamics as an underlying cause of nephrotoxicity [2–8]. Cyclosporin treatment has been shown to cause a decrease in renal blood flow thought to be mediated by changes in metabolism of vasoactive substances in the kidney [3, 5, 7–9], including an enhanced production of the potent vasoconstrictor, thromboxane [5, 7]. This concept is supported by the finding that administration of vasodilators diminishes cyclosporin-mediated nephrotoxicity [4, 9–11]. The role of renal cytochrome P-450 in the metabolism of prostaglandins has been well documented [12–16]. The effect of cyclosporin treatment on cytochrome P-450 in the kidney, however, has not been investigated.

In contrast, much research has focused on the relationship between cyclosporin and the hepatic cytochrome P-450-dependent monooxygenase system [3, 17–22]. About 90% of cyclosporin metabolism is carried out by the liver [2] to form a larger number of metabolites [17]. It has been shown that induction of hepatic cytochrome P-450 activity by various agents lowers the plasma levels of cyclosporin and the associated nephrotoxicity [3, 17, 19]. Conversely, inhibitors of cytochrome P-450 have been shown to enhance nephrotoxicity associated with elevated cyclosporin levels [20]. Moreover, in animals treated with cyclosporin, hepatic cytochrome P-450 concentration and certain P-450-dependent activities are decreased [3, 18, 21]. There is evidence suggesting that cyclosporin or its metabolites may directly inactivate hepatic cytochrome P-450 [3], and it has been reported that cyclosporin *in vitro* can inhibit mouse liver microsomal benzo[*a*]pyrene activity [22]. The mechanism by which cyclosporin decreases the concentration of cytochrome P-450 in the liver, however, has not been investigated, and it is not known whether cyclosporin decreases the concentration of the hemoprotein by altering heme metabolism activities in this organ.

The present study was undertaken to elucidate the effect of cyclosporin on renal heme metabolism and cytochrome P-450 levels, and to examine the mechanism underlying the decrease in liver cytochrome P-450 as pertains to heme metabolism.

METHODS

Male Sprague–Dawley rats (200–250 g) were purchased from Harlan Industries, Madison, WI and maintained on Purina rat chow and water *at lib.*

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Cyclosporin A and Cremophor EL were supplied by the Sandoz Co., East Hanover, NJ. Porphyrin complexes were purchased from Porphyrin Products, Logan, UT. Other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO. All chemicals were of analytical grade. Cyclosporin was diluted with 0.9% NaCl for injection. Cyclosporin is supplied in a vehicle consisting of Cremophor EL (polyoxyethylated castor oil) in ethyl alcohol, and the appropriate amounts of these constituents were also diluted in 0.9% NaCl for use as vehicle treatment. Animals were treated (s.c.) with 25 or 50 mg/kg/day of cyclosporin for 6 days, or vehicle in volumes equivalent to cyclosporin treatments. The regimen of treatments were essentially the same as that used by Sullivan *et al.* [2] and Galinsky *et al.* [21]. Control animals received saline. Animals were killed 24 hr after the last injection [2], and the liver was perfused with 0.9% saline. The liver and kidneys were homogenized in Tris–HCl buffer (0.01 M, pH 7.4) containing 0.25 M sucrose. The cellular homogenate was used for determination of porphyrin content as well as activity of ALA* synthetase. The activities of ALA dehydratase, biliverdin reductase and uro-I synthetase were measured in the cytosol. Microsomal fraction was used for measurement of activities of heme oxygenase and NADPH–cytochrome P-450 reductase, and of concentrations of heme and cytochromes P-450 and b_5. This fraction was also used for measurements of benzo[*a*]pyrene hydroxylase and aniline hydroxylase activities. Biliverdin reductase was purified from control rat liver as described earlier [23].

Assay procedures. The activity of ALA synthetase was determined spectrophotometrically as described earlier [24] by measuring absorbancy between 552 and 575 nm using an extinction coefficient of 58 $mM^{-1}\,cm^{-1}$. Total porphyrin content was measured fluorometrically by the method of Granick *et al.* [25]. Coproporphyrin I was used as the standard. The procedure described by Mauzerall and Granick [26] was used for the measurement of ALA dehydratase activity. The amount of protein in the assay medium (0.225 ml) was 2–3 mg, and the concentration of ALA was 150 mM. The enzyme activity was determined from the amount of porphobilinogen (PBG) formed. Uro-I synthetase activity was determined according to the fluorometric method of Granick *et al.* [27]. Renal cytochrome P-450 content was measured by the procedure of Jones *et al.* [28] which corrects for cytochrome oxidase contamination. The concentration of cytochrome P-450 in the liver was determined by the method of Omura and Sato [29]. Cytochrome b_5 content was measured utilizing the procedure of Estabrook and Werringloer [30]. The activity of heme oxygenase was assayed as detailed previously [31] by detecting the formation of bilirubin in the presence of biliverdin reductase. An extinction coefficient of 40 $mM^{-1}\,cm^{-1}$ was used [32]. NADPH–cytochrome P-450 reductase activity was determined by the procedure of Strobel and Dignam [33]. The activity of biliverdin reductase was measured as described earlier [23]. Microsomal benzo[*a*]pyrene hydroxylase activity was measured by the fluorometric assay of Dehnen *et al.* [34]. The formation of 3-hydroxybenzo[*a*]pyrene was measured using authentic compound as the standard. The aniline hydroxylase activity was assessed using the procedure of Imai *et al.* [35]. Protein content was measured according to the method of Lowry *et al.* [36].

All spectral studies were carried out using an Aminco–Chance DW-2C spectrophotometer. The results are expressed as means ± SD for four to eight determinations; one rat was used for each determination. The data were analyzed by Student's *t*-test, and differences were considered significant if the P value was ≤ 0.05.

RESULTS

Preliminary studies demonstrated a dose-dependent change in renal function, as determined by an increase in plasma BUN concentration, after 6 days of treatment of rats with 25 or 50 mg/kg cyclosporin. A significant change in BUN was not noted after 3 days of treatment. The observed pattern of response was consistent with the observation reported by Galinsky *et al.* [21] that cyclosporin effects on hepatic cytochrome P-450 levels are not evident until 4 daily treatments with cyclosporin. Accordingly, 6 days was selected for the duration of treatment. The comparative dose-dependent effect of cyclosporin on the content of cytochrome P-450 in liver and kidney is shown in Fig. 1. As shown, in these organs the pattern of response elicited by cyclosporin treatment was reciprocal in nature. In rats treated with 25 or 50 mg/kg cyclosporin, the hepatic cytochrome P-450 content was decreased to about 70–75% of the control (saline-treated) value. In these animals, the renal level of the cytochrome was increased to about 145% and 170% of the control value respectively. The vehicle of cyclosporin, Cremophor EL, did not affect cytochrome P-450 levels significantly in either organ when compared with the control values.

Attempts were made to determine whether changes in cytochrome P-450 content detected by spectral analysis would be reflected functionally in the rate of biotransformation of substrates. Aniline and benzo[*a*]pyrene were selected as substrates since the hepatic metabolism of those substrates has been shown to be sensitive to cyclosporin treatment [18, 22]. The data obtained with the liver drug metabolism activities were consistent with a depression of cytochrome P-450 content in this organ. In the kidney, however, the increase in cytochrome P-450 level was not accompanied by an increase in drug biotransformation activity. As shown in Fig. 2, in the liver when benzo[*a*]pyrene was used as the substrate, the rate of hydroxylation was depressed markedly, and the activity in both groups of cyclosporin-treated rats was reduced to a mere 20–28% of animals receiving the vehicle. In contrast, in the kidney of animals treated with 25 or 50 mg/kg cyclosporin, benzo[*a*]pyrene hydroxylase activity, when compared with animals receiving the vehicle, was not decreased. When the activity was compared with the control animals, in the kidney of 25 mg/kg cyclosporin-treated rats, the hydroxylase was decreased

* Abbreviations: ALA, δ-aminolevulinate; and uro-I, uroporphyrinogen-I.

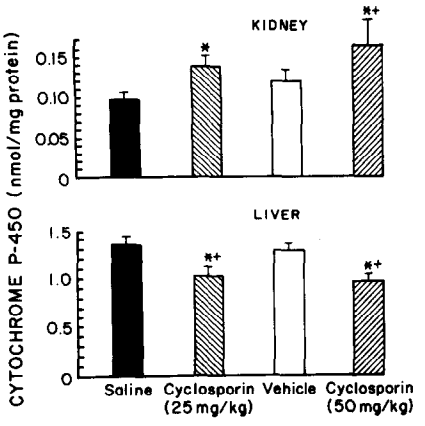

Fig. 1. Comparative influence of cyclosporin A treatment on the liver and kidney cytochrome P-450 concentration. Groups of four male Sprague–Dawley rats (200–250 g) were treated (s.c.) with 25 or 50 mg/kg cyclosporin A once daily for 6 days, or the same regimen of Cremophor EL (cyclosporin vehicle) at doses equivalent to those received by animals treated with cyclosporin. The control animals received saline. The microsomal fractions were prepared from perfused organs and used for measurement of cytochrome P-450 concentration. The liver cytochrome P-450 was measured by the method of Omura and Sato [29] and that of the kidney was measured by the procedure of Jones *et al.* [28]. The data shown are means ± SD. Significance was measured by Student's *t*-test. Key: (*) $P \leq 0.05$ when compared with the saline-treated rats; and (+) $P \leq 0.05$ when compared to the Cremophor EL-treated rats; (■) control, (□) Cremophor EL equal to the amount received by rats treated with cyclosporin, (▨) cyclosporin 25 mg/kg, and (▨) cyclosporin 50 mg/kg.

Table 1. Effect of cyclosporin A treatment on rat liver microsomal aniline hydroxylase activity

Treatment	Aniline hydroxylase (pmol/mg/min)
Saline	12.34 ± 1.37
Vehicle	12.74 ± 0.69
Cyclosporin	9.56 ± 1.02*

Groups of four male Sprague–Dawley rats (200–250 g) were treated (s.c.) with 25 mg/kg/day of cyclosporin, vehicle (Cremophor EL), or saline for 6 days. The animals were killed 24 hr after the last injection, and the microsomal fraction was prepared from the perfused livers and used for measurement of aniline hydroxylase activity. The enzyme activity was assessed from the rate of *p*-hydroxyaniline formation [35]. Values are means ± SD.

* $P \leq 0.05$ when compared with the saline-treated group (Student's *t*-test).

only modestly. The possibility was examined that the inhibition of benzo[*a*]pyrene hydroxylase activity by the liver microsomal fraction reflects a decrease in the rate of NADPH–cytochrome P-450 reductase activity. However, as also shown in Fig. 2, the reductase activity was not altered significantly in the liver of cyclosporin-treated animals when compared with the vehicle-treated rats. When compared with the saline-treated animals, the reductase activity was inhibited modestly in the animals receiving 25 mg/kg cyclosporin. In the kidney, NADPH–cytochrome P-450 reductase activity was not affected. As with benzo[*a*]pyrene hydroxylase, aniline hydroxylase activity of the microsomal fraction of the liver of animals treated with 25 mg/kg cyclosporin was also decreased (Table 1). However, the magnitude of

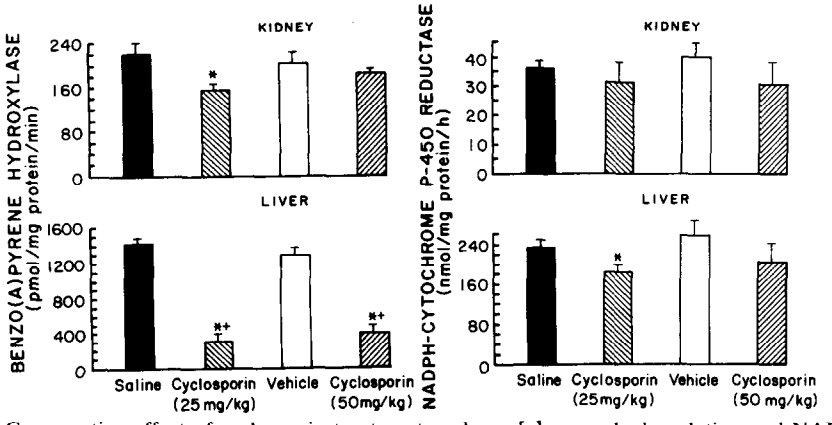

Fig. 2. Comparative effect of cyclosporin treatment on benzo[*a*]pyrene hydroxylation and NADPH–cytochrome P-450 reductase activity in the liver and kidney. Rats were treated with cyclosporin A, Cremophor EL or saline as described in the legend to Fig. 1. The microsomal fractions were prepared from perfused organs and used for measurement of enzyme activities. Benzo[*a*]pyrene hydroxylase activity was measured by the method of Dehnen *et al.* [34] and that of the reductase was assessed as described by Strobel and Dignam [33]. Data shown are means ± SD of eight determinations. One rat was used for each determination. Key: (*) $P \leq 0.05$ when compared with the saline-treated rats, and (+) $P \leq 0.05$ when compared with the Cremophor EL-treated rats; (■) control, (□) Cremophor EL equal to the amount received by rats treated with cyclosporin, (▨) cyclosporin 25 mg/kg, and (▨) cyclosporin 50 mg/kg.

Table 2. Effects of cyclosporin A treatment on the activities of δ-aminolevulinate synthetase, δ-aminolevulinate dehydratase, uroporphyrinogen-I synthetase and total porphyrin content in the rat kidney and liver

Organ	Cyclosporin treatment (mg/kg)	ALA synthetase (pmol/mg/hr)	ALA dehydratase (nmol/mg/hr)	URO-I synthetase (pmol/mg/hr)	Porphyrins (pmol/mg)
Liver	0	125 ± 41	13.3 ± 0.1	2145 ± 260	1.31 ± 0.14
	25	97 ± 23	11.4 ± 3.2	2165 ± 210	1.53 ± 0.19
	50	285 ± 84*	12.9 ± 0.6	2317 ± 203	1.20 ± 0.01
Kidney	0	302 ± 47	7.3 ± 0.5	873 ± 96	13.7 ± 4.7
	25	168 ± 63*	5.8 ± 0.2*	727 ± 148	4.5 ± 2.1*
	50	223 ± 37	4.3 ± 0.5*	677 ± 119	4.7 ± 0.6*

Male Sprague–Dawley rats (200–250 g) were treated (s.c.) daily with the above indicated doses of cyclosporin, for 6 days, and killed 24 hr after the last injection. The organs were perfused *in situ*, and the subcellular fractions were prepared as described in Methods. Cellular homogenate was used for measurement of ALA synthetase and ALA dehydratase activities and porphyrin content. The cytosol fraction was used for measurement of uro-I synthetase activity. Measurements were carried out as detailed in the text. The data shown are means ± SD of four to eight determinations. Cremophor EL did not elicit a significant response in any of the above measured variables. One rat was used for each determination.

* $P \leq 0.05$ when compared with the control value.

decrease was by far less than that noted with the benzo[*a*]pyrene hydroxylase activity; aniline hydroxylation was reduced to about 77% of the control value. Due to the low level of aniline hydroxylase activity in the kidney, the activity could not be measured accurately by the presently used colorimetric procedure.

Further studies were then carried out to determine the cellular basis for the alteration in cytochrome P-450 levels in the liver and the kidney, as pertain to heme biosynthesis and degradation. The effects of cyclosporin treatment on several key enzymes of heme and porphyrin biosynthesis were assessed, and the results are shown in Table 2. A striking effect of cyclosporin treatment (25 mg or 50 mg/kg) on the kidney porphyrin levels was noted. As shown, both doses of cyclosporin profoundly decreased porphyrin concentration (to 30% of the control) in the kidney. Porphyrin levels were not affected in the liver. Other differences were also noted between the two organs in the response of heme biosynthesis enzymes to cyclosporin. In the liver, the activity of the rate-limiting enzyme of heme synthesis, ALA synthetase, was unaffected by the lower dose of cyclosporin, but was significantly elevated in response to the higher dose of the drug. The conversion of ALA to the monopyrrole, porphobilinogen, is catalyzed by ALA dehydratase. The condensation of 4 porphobilinogen molecules to uroporphyrin is catalyzed by uro-I synthetase. As shown in the liver, the activities of ALA dehydratase and uro-I synthetase were refractory to treatment with either dose of cyclosporin. In the kidney, cyclosporin at the dose of 25 mg/kg resulted in a decrease of ALA synthetase activity to about 60% of the control value; the higher dose of the drug also decreased the synthetase activity, but the decrease did not reach statistical significance. Also in the kidney, activity of ALA dehydratase was significantly inhibited by both doses of cyclosporin; uro-I synthetase activity was not significantly affected by either regimen of cyclosporin treatment. Cremophor EL treatment did not affect any of the variables assessed in Table 2 (data not shown).

Clearly, an inhibition of heme biosynthesis in the liver and an increase in the synthesis of heme in the kidney did not account for the above noted changes in cytochrome P-450 levels in these organs. Therefore, the effect of cyclosporin on heme degradation pathway was assessed, and the results are depicted in Fig. 3. Heme oxygenase is the rate-limiting enzyme in this pathway and is readily induced by a wide variety of chemical stimuli. As with the cytochrome P-450-dependent monooxygenase activities, the activity of this enzyme requires the concerted activity of NADPH–cytochrome P-450 reductase. Biliverdin reductase is the terminal enzyme in the pathway, and its activity results in formation of bilirubin. As shown, cyclosporin treatment (25 or 50 mg/kg) did not significantly affect heme oxygenase activity in the liver. Similarly, the same regimens of cyclosporin treatments did not alter the activity of biliverdin reductase in the liver. In contrast, in the kidney heme oxygenase activity was significantly inhibited in response to treatment with both doses of cyclosporin (to 65–70% of control values). Biliverdin reductase was not depressed by cyclosporin treatment in this organ.

The possibility was examined whether the noted increase and decrease, respectively, in the cytochrome P-450 in the kidney and the liver of cyclosporin-treated animals reflected a generalized effect of the drug on the microsomal hemoproteins. Accordingly, the concentration of cytochrome b_5 in the liver and kidney microsomal fractions was measured. The values obtained for the liver cytochrome b_5 were 0.63 ± 0.02 vs 0.62 ± 0.02 nmol/mg protein for the control and the cyclosporin-treated (25 mg/kg) animals, respectively. The values obtained for the kidney cytochrome b_5 were 0.092 ± 0.008 vs 0.086 ± 0.020 for the control and cyclosporin-treated rats, respectively. These results suggest the selective response of cytochrome P-450

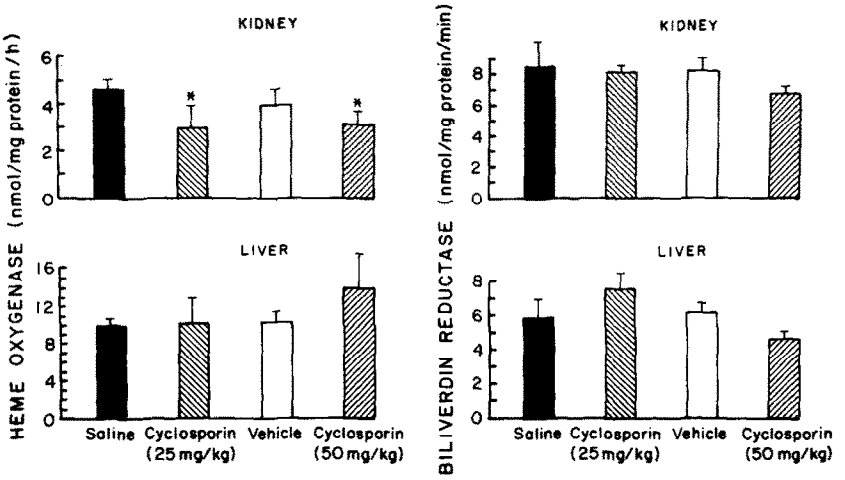

Fig. 3. Comparative effect of cyclosporin A on the activities of heme oxygenase and biliverdin reductase in rat liver and kidney. Male Sprague–Dawley rats (200–250 g) were treated with cyclosporin as described in the legend to Fig. 1. The microsomal and the cytosol fractions were prepared from perfused organs. The microsomes were used for measurement of heme oxygenase, and the cytosol fraction was used for measurement of biliverdin reductase activity. Experimental details are provided in Methods. The data shown are means ± SD of eight determinations. One rat was used for each determination. Key: (*) $P \leq 0.05$ when compared with the control value; (■) control; (□) Cremophor EL equal to the amount received by rats treated with cyclosporin, (▨) cyclosporin 25 mg/kg, and (▨) cyclosporin 50 mg/kg.

to cyclosporin.

DISCUSSION

The present study demonstrates that in the kidney enzymes of heme biosynthesis and degradation pathways display a remarkable degree of sensitivity to cyclosporin. For the most part, however, these activities were unaffected in the liver. The most striking observation with the kidney was the dramatic decrease in the renal content of porphyrins. In our experience, we are not aware of any other chemical, with the exception of *cis*-platinum, that causes such a severe depression in renal porphyrin levels [37]. It is noteworthy that *cis*-platinum is also known for its nephrotoxicity. Aside from the similarity of their effects on renal porphyrin concentration, cyclosporin and *cis*-platinum resemble each other in their abilities to cause an increase in cytochrome P-450 in the face of a depressed tissue concentration of porphyrins, and inhibition of certain enzymes of heme biosynthesis. At this time, the relevance of depressions in porphyrin levels and activities of heme biosynthesis enzymes in the kidney to the nephrotoxicity of cyclosporin and *cis*-platinum is not evident. However, since porphyrins are precursors for the production of hemoproteins, including the respiratory cytochromes, a severe decrease in their production could result in deficiencies in cellular respiration and energy production. This, in turn, could exacerbate and/or contribute to the toxicity of the drugs.

The finding that cyclosporin did not alter significantly either hepatic heme biosynthesis or degradation suggests that cyclosporin-mediated decrease of hepatic cytochrome P-450 concentration has involved factors such as the direct action of the drug on the hemoprotein, or its effect on regulation of synthesis and/or degradation of the apoprotein moiety of the cytochrome. The finding that cytochrome b_5 concentration in the liver of cyclosporin-treated rats was not significantly altered is consistent with the apparent lack of perturbations in heme metabolic activities in this organ. The present finding confirms the reports of cyclosporin-mediated decreases in hepatic cytochrome P-450 content [3, 18, 21], and demonstrates its ability to rather selectively inhibit biotransformation of certain substrates. The latter conclusion is based on the observation that the extent of inhibition of benzo[*a*]pyrene and aniline hydroxylation notably differed. The apparent selectivity is consistent with reported findings by Galinsky *et al.* [21]. These investigators measured cytochrome P-450-dependent drug-metabolizing activities in the rat liver following cyclosporin treatment; although a significant decrease in total cytochrome P-450 contribution was observed, cytochrome P-450-dependent biotransformation activities were decreased selectively.

At this time it is not clear whether cyclosporin-mediated increase in renal cytochrome P-450 contributes to the nephrotoxicity of the drug or if other toxic events caused by cyclosporin lead to the increase. The former possibility is, however, consistent with the following reported findings. In the kidney, cytochrome p-450, for the most part, is associated with biotransformations of endogenous compounds such as vitamin D, prostaglandins, leukotrienes and fatty acids [13–16, 38, 39] rather than metabolism of drugs, such as cyclosporin [2, 17]. For instance, thromboxane synthetase, which catalyzes the formation of thromboxane A_2, a potent

vasoconstrictor, is a form of cytochrome P-450 [10], and its production is stimulated in chronic cyclosporin nephrotoxicity [5, 7]. Cyclosporin has been reported to adversely affect renal hemodynamics [4–9, 40], and its nephrotoxic effects can be diminished by coadministration of vasodilators [4, 9–11]. In view of these reports, the presently observed increase in renal cytochrome P-450 may well be expected to alter cytochrome P-450-dependent functions in the kidney. The possible alteration in prostaglandin biotransformation potentially could be most intimately linked to the nephrotoxicity of the drug.

Availability of heme for production of cytochrome P-450 as well as other hemoproteins is controlled by both the activities of heme biosynthetic pathway enzymes as well as the heme degradation enzymes. There is often a reciprocal relationship between the rate of heme oxygenase activity and the cytochrome P-450 level in the liver [41]. Moreover, we have recently shown that heme oxygenase can utilize certain isozymes of cytochrome P-450, as well as cytochrome P-420, as the substrate [42]. The finding that in the kidney cyclosporin treatment inhibited both the synthesis and degradation of heme, yet the concentration of cytochrome P-450 was increased, suggests the key role of heme oxygenase in maintenance of the hemoprotein levels in this organ. It is noteworthy that the presently reported inhibition of heme oxygenase by cyclosporin in the kidney is rather unusual. Heme oxygenase is the rate-limiting enzyme of heme degradation, and its activity is influenced by a wide variety of substances. However, in nearly all reported instances its activity is induced rather than inhibited [41, 43]. To date, the only known agents capable of inhibiting heme oxygenase are synthetic metalloporphyrins, which act as competitive inhibitors for binding the physiological substrate [41, 43]. Since cyclosporin is structurally dissimilar to metalloporphyrin, it constitutes a novel type of compound that can down-regulate heme oxygenase.

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