Cyclosporine suppresses rat hepatic cytochrome P450 in a time-dependent manner

LANE J. BRUNNER, WILLIAM M. BENNETT, and DENNIS R. KOOP

Pharmaceutics Division, College of Pharmacy, The University of Texas at Austin, Austin, Texas; Division of Nephrology, Department of Medicine, and Department of Physiology and Pharmacology, Oregon Health Sciences University, Portland, Oregon, USA

Cyclosporine suppresses rat hepatic cytochrome P450 in a time-dependent manner.

Background. Cyclosporine is a potent immunosuppressant known to selectively suppress specific cytochrome P450 (P450) isoforms following chronic therapy in the rat. Cyclosporine undergoes significant hepatic metabolism in the rat, primarily due to P450 3A isoforms. Hence, alterations in hepatic metabolism of cyclosporine may lead to changes in drug pharmacokinetics or pharmacodynamics. The purpose of this study was to examine the temporal effect of chronic cyclosporine dosing on P450 protein expression and metabolic activity in a rat model of chronic cyclosporine nephropathy.

Methods. Adult male rats were administered cyclosporine 15 mg/kg/day or vehicle 1 ml/kg/day by subcutaneous injection for up to 28 days. To examine whether or not metabolic activity recovered following drug removal, additional rats were administered cyclosporine for 28 days followed by vehicle for up to an additional 15 days. Hepatic P450 protein expression and microsomal metabolic activity were measured by Western blot analysis and in vitro steroid hydroxylation, respectively.

Results. Cyclosporine trough levels progressively increased over the 28 days period and were still measurable for up to 15 days after discontinuation. Immunoblot analysis indicated that chronic cyclosporine treatment suppressed P450 3A2 expression and in vitro steroid hydroxylation in a time-dependent manner. Fifteen days following discontinuation of cyclosporine dosing, hepatic metabolic activity and microsomal P450 3A2 levels returned to near pre-dosing levels.

Conclusions. We conclude that the time-dependent P450 suppression by cyclosporine may at least partially explain the variability in cyclosporine metabolism during chronic treatment in the rat.

Cyclosporine is a cyclic undecapeptide of fungal origin used for the prevention of rejection in solid organ transplants and for graft-versus-host disease prophylaxis in allogeneic bone marrow transplantation. Despite its successful use, cyclosporine therapy is hampered by significant dose-limiting toxicities to the kidney, liver, and central nervous system [1, 2]. While these toxicities are related to circulating cyclosporine concentrations, they are not always predictable.

The major pathway for cyclosporine elimination is through hepatic metabolism by cytochrome P450 (P450) [3, 4]. Although over 25 metabolites have been identified [5], three predominant products are the result of either hydroxylation or demethylation pathways. These metabolic pathways appear to be similar between many species, including humans and rodents [6]. The P450 3A family is known to be responsible for the majority of cyclosporine metabolism [7], although other P450 enzymes have been implicated as well [8,9]. Modulation of hepatic P450 levels has been shown to markedly affect cyclosporine blood levels and toxicity [10, 11]. Induction of hepatic P450 enzymes results in a decrease in circulating cyclosporine levels and decreased drug-associated toxicities. Conversely, inhibition of hepatic P450 may result in ciclosporine toxicity.

We have recently shown that cyclosporine selectively suppresses P450 protein expression and metabolic activity following chronic administration in the rat [8]. The affected P450 enzymes were were the male-specific isoforms P450 3A2 and 2C11. Following 28 days of cyclosporine administration, male rats showed no detectable hepatic microsomal P450 3A2 or 2C11 protein and significantly less in vitro metabolic activity as compared with gender-matched controls. Despite this, whole blood cyclosporine trough levels were nearly half the values found in cyclosporine-treated female rats. Thus, enzymes other than P450 3A2 are likely to be involved with cyclosporine metabolism. Indeed, recent evidence suggests that a new female-predominant inducible P450 isoform, P450 3A9, may be involved with drug metabolism [12].

The present study was conducted to determine whether the suppression of P450 isoforms by cyclosporine was dependent on the length of drug treatment, or whether the suppression occurred close to the initiation of drug administration. We examined the time-dependent effect in our
METHODS

Animals

Sixty male ten-week-old Sprague-Dawley rats were purchased from Charles River Breeders (Wilmington, MA, USA) and individually housed in wire-bottom cages in a 12-hour light/dark cycle animal facility with controlled humidity and temperature. Following a five-day acclimation period, rats were pair-fed a low-salt rice diet (8.5% protein, 76.6% carbohydrate, 4.3% fat, 0.05% salt, enriched with ferric orthophosphate and thiamin mononitrate; Harlan Teklad, Indianapolis, IN, USA) and allowed free access to tap water [8, 13]. Prior to the initiation of the study, approval of the protocol was given by the Institutional Animal Use and Care Committee and all animal procedures followed the Principles of Laboratory Animal Care published by the National Institutes of Health.

Materials

Cyclosporine was provided in powder form (Sandoz Research Institute, East Hanover, NJ, USA) and dissolved in ethanol. The solution was diluted to 12.5% with olive oil (12.5% vol/vol with olive oil) to a final cyclosporine concentration of 15 mg/ml. Furosemide was purchased from American Reagent Laboratories, Inc. (Shirley, NY, USA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XII), NADP (grade III), and testosterone were purchased from Sigma Chemical Co. in the highest purity available. Goat anti-rabbit IgG, rabbit peroxidase anti-peroxidase, and goat anti-rabbit horseradish peroxidase were purchased from Organon Teknika (Durham, NC, USA).

Drug treatment

Following the acclimation period, rats were given a single intraperitoneal dose of furosemide (4 mg/kg) to initiate salt depletion and started on the low-salt rice diet. After one week, weight-matched pairs of rats were administered once daily subcutaneous doses of cyclosporine 15 mg/kg or vehicle 1 ml/kg. One group of rats (N = 4) was not administered drug or vehicle and thus represented Day 0 of the study. Additional rats were administered cyclosporine or vehicle (N = 4 each group) for 3, 7, 14, 21, or 28 days. Two additional rat groups (N = 4 each) were administered cyclosporine 15 mg/kg 28 days and then administered vehicle 1 ml/kg only for an additional 7 or 15 days. The final two rat groups (N = 4 each) were administered vehicle 1 ml/kg for a total of 35 or 43 days. Cyclosporine and vehicle were administered at the same time each day to minimize chronobiologic variability in drug toxicity [14]. Daily body weights were measured. On the final day of drug or vehicle dosing, tail blood pressures were measured (Natsume Model KN-210-1; Peninsula Laboratories, Inc., Belmont, CA, USA). The rats were then placed into standard rodent metabolic cages (Nalge, Rochester, NY, USA) for passive urine collection for 24 hours. After the urine collection period, rats were anesthetized with a single intraperitoneal dose of ketamine 100 mg/kg. Intravenous blood samples were collected into glass tubes containing EDTA for measuring cyclosporine concentrations. An aliquot of blood was centrifuged at 3000 × g for 15 minutes and the plasma was used to measure potassium. A single blood sample was collected and allowed to clot at room temperature and the serum was separated by centrifugation at 3000 × g for 15 minutes and was used to measure creatinine levels. Animals were then killed with a single lethal intravenous injection of ketamine 200 mg/kg. The livers were removed for the determination of hepatic microsomal activity.

Microsome isolation

Rat livers were immediately excised following sacrifice, placed into liquid nitrogen, and stored at −70°C. Microsomes were isolated from livers by differential centrifugation as previously described [15]. Samples were maintained at 4°C during the microsome preparation. In brief, aliquots of liver tissue were homogenized in four volumes of Tris chloride buffer, pH 7.4, containing 150 mM potassium chloride and 1 mM EDTA, with a tissue homogenizer (Brinkman Instruments Company, Westbury, NY, USA). Samples were then centrifuged at 10,000 × g for 20 minutes at 4°C. The supernatant was collected and centrifuged at 211,000 × g for 40 minutes at 4°C. The supernatant was discarded and the pellet was resuspended and washed in sodium pyrophosphate buffer, pH 7.4, containing 1 mM EDTA with a Potter-Elvehjem tissue grinder. The suspension was centrifuged again at 211,000 × g for 40 minutes at 4°C. The supernatant was discarded and the washed pellet was resuspended in a tris chloride buffer, pH 7.4, containing 20% glycerol, with a ground glass tissue grinder. Microsomes were stored at −70°C prior to analysis.
7.4, 0.2 mg microsomal protein, 250 μM testosterone in methanol (final concentration did not exceed 0.7% vol/vol), and an NADPH regeneration system consisting of 0.5 mM NADP, 10 mM glucose-6-phosphate, 10 mM magnesium chloride, and 5 U glucose-6-phosphate dehydrogenase. Total reaction volume was 1 ml. Mixtures were preincubated at 37°C for three minutes and reactions were initiated by the addition of glucose-6-phosphate dehydrogenase. Incubations proceeded for 15 minutes and were quenched by the addition of 5 ml of dichloromethane. The internal standard (3.6 nmol of 11α-hydroxyprogesterone) was added and the samples mixed. The organic layer was removed and dried under reduced pressure. Dried extracts were dissolved in 200 μl methanol and stored at 4°C until analyzed. Samples were stable for at least four months at 4°C.

Steroid 5α-reductase activity was measured by the metabolic conversion of androstenedione to androstanedione in vitro. The reaction is analogous to the testosterone hydroxylase reaction described above except that (1) androstenedione 250 μM was used as the substrate, (2) 60.5 nmol of internal standard (11α-hydroxyprogesterone) was added; (3) incubation time was 20 minutes; and (4) the samples were stable for at least two months.

**Chromatography**

Testosterone and metabolites were separated and quantified by high pressure liquid chromatography (HPLC). In brief, 20 μl of the extracts were injected on a Waters HPLC system consisting of a Model U6K injector with, dual Model 501 solvent pumps, and a Model 484 variable wavelength detector (Millipore Corporation, Milford, MA, USA). Metabolites were resolved at 40°C (Model CH-30 solvent pumps, and a Model 484 variable system consisting of a Model U6K injector with, dual solvent pump, variable wavelength detector, and gradient program). Compounds were separated using a concave gradient (curve 8) from 90% solvent A (methanol:water:acetonitrile, 39:60:1) to 85% solvent B (methanol:water:acetonitrile, 80:18:2) was delivered over 22 minutes at 1.5 ml/min. A minimum eight-minute washout of 90% solvent A preceded each analysis. Absorbance was monitored at 238 nm. A Baseline 810 Chromatography Workstation (Millipore Corporation, Milford, MA, USA) was used for gradient delivery, data acquisition, and peak analysis. Testosterone metabolites were quantitated by comparison of peak area ratios (metabolite:internal standard) with those generated with authentic standards. Rates were determined under conditions that were linear with protein and time.

Androstenedione and reduced metabolites were separated and quantified by HPLC using the same HPLC system described above. Compounds were resolved at ambient temperature on a 250 mm × 4.6 mm C-18 column (Microsorb MV; Rainin Instrument Co., Woburn, MA, USA) preceded by a 10 mm × 4.3 mm C-18 guard column (Upchurch Scientific, Oak Harbor, WA, USA). An isocratic solvent (75% methanol, 25% water) was delivered over 12 minutes at 1 ml/min. Absorbance was monitored at 290 nm. Data were collected and analyzed in a manner similar to that for testosterone described above.

**Gel electrophoresis and immunoblot analysis**

SDS-PAGE was performed as previously described [16] with an 8% polyacrylamide separating gel. Gibco BRL prestained high range molecular weight markers (Life Technologies, Gaithersburg, MD, USA) were used for molecular weight estimation. Protein was electrophoretically transferred to nitrocellulose sheets (Schleicher and Schuell, Keene, NH, USA) using a Genie Electrophoretic Blotter (Idea Scientific Co., Minneapolis, MN, USA) as previously described [17]. Rabbit anti-human P450 3A4 antibody was generously provided by Dr. Kenneth E. Thummel (University of Washington, Seattle, WA, USA) and rabbit anti-rat P450 2C11 antibody was generously provided by Dr. Edward T. Morgan (Emory University, Atlanta, GA, USA). For the detection of P450 3A4 immunoreactive proteins, nitrocellulose sheets were blocked with 3% NFDM in TBS and incubated with 2.5 μg/ml rabbit anti-human P450 3A4 antibody (in 3% NFDM in TBS) then a 1:10,000 dilution of goat anti-rabbit IgG (in 3% NFDM in TBS), and finally a 1:10,000 dilution of rabbit peroxidase-anti-peroxidase (in 3% NFDM in TBS). For the detection of P450 2C11 immunoreactive proteins, nitrocellulose sheets were incubated with a 1:10,000 dilution of rabbit anti-rat P450 2C11 antibody (in 3% NFDM in TBS) then a 1:5000 dilution of goat anti-rabbit horseradish peroxidase (in 3% NFDM in TBS). Intermediate washes with TBS containing 0.05% Tween 20 were as previously described [17]. Immune complexes for P450 3A4 and P450 2C11 immunoreactive proteins were detected with chemiluminescence using an ECL detection kit as described by the manufacturer (Amersham, Arlington Heights, IL, USA) using Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY, USA). Immunoreactive protein band density was by direct chemiluminescence detection (Molecular Imager; Bio-Rad).

**Other assays**

Urine and serum potassium concentrations were measured by flame photometry (Instrumentation Laboratories, Lexington, MA, USA). Urine and serum creatinine, and urine osmolality were measured by a Cobas autoanalyzer (Roche Diagnostics, Nutley, NJ, USA). Whole blood cyclosporine levels were determined by monoclonal radioimmunoassay (Sandoz Research Institute, East Hanover, NJ, USA). Microsomal protein concentrations were measured using the Folin-phenol reagent [18] with bovine serum albumin as a standard.
Statistical analysis

Differences between rat groups were compared using two-factor analysis of variance. If a significant difference in the treatment effect over time was found in the analysis of variance model, then critical differences were determined by multiple contrast analysis (SuperANOVA; Abacus Concepts, Berkeley, CA, USA). Data are presented as mean ± standard error. Differences were considered significant when the probability of chance explaining the results was reduced to less than 5% (P < 0.05).

RESULTS

All animals completed the study. The mean total body weight of the rats at the beginning of the study was 327 ± 6 g for control groups as compared with 331 ± 6 g for cyclosporine-treated groups (P > 0.05). All rats gained weight over the study period and there was no significant effect of drug treatment or length of treatment on weight gain (data not shown). A summary of the effect of drug treatment on markers of renal function is given in Figure 1. Renal function was significantly reduced by the end of cyclosporine dosing. This reduction was most evident beginning 14 days after the start of cyclosporine dosing and appeared to recover to baseline values 15 days after the discontinuation of cyclosporine. Aspartate aminotransferase, a marker of drug-associated hepatotoxicity, was not statistically different between control and cyclosporine-treated rats during the study period (data not shown).

The regio- and stereospecific hydroxylation of testosterone in vitro by hepatic microsomes is an effective marker of different P450 isoforms. A summary of the effect of cyclosporine treatment on microsomal metabolism of testosterone is shown in Figure 2 (upper panels). Chronic cyclosporine treatment significantly reduced the production of both 6β-OHT and 2α-OHT in a time-dependent manner (P < 0.05).

In order to monitor the presence of specific P450 isoforms, immunoblot analysis of hepatic microsomal protein was performed (Fig. 2, lower panels). Microsomal protein from cyclosporine-treated rats showed a decrease in a single immunoreactive band, consistent with P450 3A2 during drug treatment (P < 0.05). However, P450 3A2 returned to control levels 15 days after the discontinuation of cyclosporine dosing. Similar results were found for the detection of P450 2C11 in hepatic microsomes.

The reduction of androstenedione to androstanedione by steroid 5α-reductase, a female-specific enzyme in the rat, has been used to determine the degree of feminization of male rats [8, 19]. Steroid 5α-reductase activity in cyclosporine-treated rats was significantly increased during drug treatment and returned to control levels following the discontinuation of cyclosporine treatment (P < 0.05; Fig. 3).

Whole blood cyclosporine trough levels increased during the first two weeks of drug treatment and remained constant until the end of dosing (Fig. 4). However, after cyclosporine dosing was stopped, the drug could be detected in whole blood until the end of the study.

DISCUSSION

A clear relationship between cyclosporine dose, blood levels, and toxicity or pharmacodynamic effects does not
exist in transplant patients. This lack of correlation may be due to the multitude of concurrent medications as well as changes in the patients end organ failure over time. Even after receiving a constant cyclosporine dosage, often blood level monitoring demonstrates an increase in steady-state concentrations in this patient group [20, 21]. While this increase has been attributed to improved bioavailability over time, the direct effect of cyclosporine on hepatic metabolism has received little consideration. In the present study, we investigated the effect of chronic cyclosporine administration on hepatic cytochrome P450-mediated drug metabolism in a rat model of chronic cyclosporine nephropathy. We found that cyclosporine suppressed in vitro hepatic microsomal activity as well as hepatic enzyme levels in a time-dependent manner. These suppressive effects resulted in increased blood levels of cyclosporine with chronic administration.

In a prior study, we reported the effect of chronic cyclosporine administration to male and female rats maintained on a low salt diet [8]. The study examined how 28 days of cyclosporine dosing would affect hepatic P450 protein expression and metabolic activity. At the end of the study, male rats had no detectable hepatic microsomal P450 3A protein. Microsomal metabolic activity was also significantly reduced as compared with controls. Moreover,
hepatic microsomal P450 2C11 protein, another male-specific P450 isoform, was also absent. Female rats, despite the absence of detectable P450 3A isoforms, had steady-state cyclosporine blood levels that were approximately one-half those of their male counterparts, indicating that P450-dependent cyclosporine clearance was greater in the females and that P450 enzymes other than P450 3A may be involved with cyclosporine metabolism. This study shows a time-dependent nature of reduction in P450 metabolism and further supports a role for isoforms other than P450 3A2 in cyclosporine metabolism over time.

In the present study, rats administered cyclosporine developed significant nephrotoxicity as measured by increases in serum creatinine and decreases in 24 hours creatinine clearance (Fig. 1). Within 14 days of cyclosporine administration, drug-treated rats showed significant signs of nephrotoxicity that remained until one week after the discontinuation of drug treatment. Thus, the functional damage caused by cyclosporine in this model appears to be reversible after removal of the drug. However, previous work using this model has shown that the structural damage that occurs after four weeks of cyclosporine treatment is not reversible [8, 13]. Further evidence of nephrotoxicity is shown by the increases in fractional excretion of potassium (FEK) 14 days after the start of cyclosporine dosing and the significant decrease in urinary osmolality after one week (Fig. 1).

Many drugs and natural products modulate hepatic drug metabolism through their action on P450 isoforms. While this modulation may lead to significant drug interactions, this modulation may also be exploited for therapeutic benefit [22–26]. In rats, cyclosporine has been shown to alter hepatic microsomal activity as well as hepatic protein expression [8, 27, 28]. In microsomes isolated from rats administered cyclosporine over the study period, there was a rapid decrease in both of the male-specific isoforms, 2C11 and 3A2 (Fig. 2). This suppression of protein expression was sustained during the period that cyclosporine was being given. However, two weeks after the discontinuation of cyclosporine, P450 2C11 and 3A2 protein expression returned to control levels. A similar pattern was noted when measuring in vitro hepatic microsomal catalytic activity, using the production of 6β-OHT and 2α-OHT as markers of P450 3A2 and 2C11 metabolic activity, respectively [29, 30].

During the first week of cyclosporine or vehicle dosing, there is a notable drop in hepatic microsomal P450 2C11 protein expression and catalytic activity (Fig. 2). Following this initial drop, microsomal levels of P450 3A2 and 2C11 recover to pre-drug treatment levels in rats given vehicle only. However, the microsomal P450 protein levels and catalytic activities continue to decrease in rats administered cyclosporine. The cause of this initial drop in hepatic protein P450 levels is not clear and may be due to the initial effects of the low salt diet or due to vehicle administration. In either case, rats appear to recover from this depression in activity, despite the continuation of both diet and vehicle administration. Therefore, this reduction appears to be separate from a cyclosporine-induced effect. While reports in the literature have examined the effect of changes in dietary salt or oil intake, no studies have examined the combined effect of both agents. The effect of increased dietary salt resulted in an increase in renal microsomal P450 in rats [31] as well as intestinal P450 3A activity in humans [32]. However, few data are available about the effect of salt restriction on P450 protein expression or metabolic activity. Dietary supplements of lipids were shown to increase P450 content and activity in livers of rats [33, 34]; however, the amounts of dietary lipids given were much greater than those used in the present study. Thus, it is not likely that either agent alone would account for the initial suppression of hepatic microsomal P450 seen in the present study.

The mechanism of the cyclosporine-induced suppression of hepatic P450 remains unclear, but may be related to alteration in endocrine function. Regulation of P450 3A2 and 2C11 in adult rats is governed by the secretion pattern of growth hormone [35, 36]. A high-amplitude, low-frequency secretion pattern is indicative of male-specific hepatic P450 protein expression, whereas a low-amplitude, high-frequency secretion pattern is characteristic of female-specific protein expression. Our previous work suggested that an alteration in this growth hormone secretion may be responsible for the “feminization” of the male rats administered cyclosporine for 28 days [8]. Rats that were administered cyclosporine had a significant increase in the female-specific hepatic enzyme 5α-reductase. Levels of this enzyme were significantly elevated after 14 days of cyclosporine treatment and peaked at 28 days after the start of dosing (Fig. 3). Following the discontinuation of cyclosporine dosing, the activity of 5α-reductase returned to the control level. Results from the present study are consistent with cyclosporine altering the release pattern of growth hormone in male rats making it similar to female rats. The slow, progressive effect of cyclosporine treatment on hepatic P450 protein expression and catalytic activity suggests that the effects were not immediate on the protein, but involves another mechanism.

The effect of chronic cyclosporine treatment on its own steady-state blood levels is shown in Figure 4. The increase in cyclosporine levels corresponds to the decrease in hepatic microsomal protein expression and in vitro metabolic activity. Furthermore, the increased cyclosporine levels correspond to the development of renal dysfunction in these rats. Since the half-life of cyclosporine following single dose and acute administration in the rat is approximately eight hours [14, 37], it is apparent that during the first two weeks of dosing, rats administered cyclosporine are not truly at steady state. This is consistent with the
pattern of suppression of hepatic P450 3A2 seen in the rats in this study. It appears that after two weeks of cyclosporine administration, the rats have established a new steady state, which is indicative of a decrease in hepatic P450 3A2 metabolic activity. While there is not necessarily an analogy to the regulation of P450 in humans, the present study does provide some insight into a possible mechanism for the progressive changes in steady-state cyclosporine levels often seen in transplant patients.

In summary, results from the present study show that chronic cyclosporine administration in a rat model of drug-associated nephropathy causes a significant, progressive suppression of hepatic microsomal P450 isoforms along with a corresponding decrease in microsomal catalytic activity. This suppression causes an increase in cyclosporine steady-state blood levels and the progression of renal dysfunction. The cause of this change in hepatic metabolic activity may possibly involve an alteration in circulating growth hormone levels or other endocrine regulation of hepatic microsomal P450 in the rat. Furthermore, a role for metabolic pathways other than P450 3A2 is suggested. Studies are ongoing to address the mechanism and clinical relevance of these time-dependent changes in cyclosporine metabolism.

ACKNOWLEDGMENTS

This work was presented in part at the 1997 International Congress of Nephrology in Sydney, Australia. This study was funded in part by grants from the Oregon Health Sciences University Nephrology Fund (W.M.B.) and National Institute on Alcohol Abuse and Alcoholism AA08608 (D.R.K.). The authors gratefully acknowledge the technical assistance of Jessie Lindsey and Karen M. Hausman.

Reprint requests to Lane J. Brunner, Ph.D., Pharmaceutics Division, PHR 4214E, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712-1074, USA.

E-mail: ljb@mail.utexas.edu

REFERENCES

12. MAJNKE A, STROTKAMP D, ROOS PH, GANSTEIN WG, CHABOT GG, NEF P: Expression and inducibility of cytochrome P450 3A9 (CYP3A9) and other members of the CYP3A subfamily in rat liver. Arch Biochem Biophys 337:62–68, 1997
21. HALPERT JR, GUENGERICH FP, BENDR CORRELA MA: Contempo-


