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Research paper

Effect of dose on cyclosporine-induced suppression of hepatic cytochrome P450 3A2 and 2C11

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Abstract

Cyclosporine is a potent immunosuppressive drug that undergoes extensive hepatic metabolism catalyzed primarily by the cytochrome P450 (P450) 3A enzyme family. Cyclosporine alters its own metabolism by selective suppression of specific P450 isoforms after chronic therapy in rats. Modulation of hepatic P450 by chronic cyclosporine dosing is associated with increased blood concentrations leading to nephropathy. However, the relationship between cyclosporine dose and hepatic enzyme suppression is not known. The purpose of this study was to examine the effect of escalating doses of cyclosporine on P450 regulation and metabolic activity in the rat. Following 1 week of a low-salt diet, rats were given cyclosporine 5, 15, 30 or 50 mg/kg per day or an equal volume of vehicle for 2 weeks via oral gavage. At the end of the dosing period, livers were removed and hepatic microsomes prepared. Hepatic P450 proteins were measured using Western blot analysis and catalytic activity determined by in vitro testosterone hydroxylation. Cyclosporine dosing suppressed both P450 3A2 and 2C11 protein expression and catalytic activity in a dose-dependent manner. Catalytic activity of two other P450 isoforms, 2A1 and 2B1, were unchanged by cyclosporine administration. Thus, the selective suppression of hepatic microsomal P450 by cyclosporine is not only dependent on the length of therapy, but also the dose administered. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cyclosporine; Cytochrome P450; Hepatic; Metabolism; Rat

1. Introduction

Cyclosporine is a potent immunosuppressant of fungal origin that is widely used for the prevention of rejection following allograft transplantation and graft-vs.-host disease prophylaxis after marrow transplantation [1–3]. Despite its utility, cyclosporine therapy is complicated by significant dose-limiting toxicities to the kidney, liver, and central nervous system. Although these toxicities appear to be dependent on dose and circulating cyclosporine concentrations, prediction of toxicity or efficacy is often difficult.

The primary pathway of cyclosporine elimination is through hepatic metabolism by the cytochrome P450 (P450) enzyme system [4,5]. Cytochrome P450 is a superfamily of genes whose products represent a divergent group of enzymes related by substrate similarity and amino acid sequence [6]. Currently there are more than 480 different P450 enzymes encoded by more than 70 different gene families in both eukaryotic and prokaryotic species. Despite this large number of enzymes, only a few have been shown to be of importance for the metabolism of therapeutic agents. Even though P450 enzymes are located in numerous tissues, those enzymes located in the liver and small intestine have received the most attention regarding drug absorption and elimination and their subsequent role in therapeutics.

The metabolic pathway of cyclosporine is similar between humans and rodents [7]. Cyclosporine metabolism in the small intestine and liver is primarily by *N*-demethylation and hydroxylation reactions [8–10] to three major oxidative metabolites: the 4-*N*-desmethylated metabolite (AMN4), the monohydroxylated 1-β metabolite (AM1), and the 9-γ-hydroxylated metabolite (AM9). The production of these metabolites is chiefly catalyzed by members of the P450 3A family but also other P450 enzymes are thought to be involved [11]. Male and female juvenile rats constitutively express hepatic P450 3A2, however, upon sexual maturation, only male rats have detectable levels of P450 3A2 protein in liver tissue [12]. Thus, hepatic metabolism of certain substrates in rats is age-dependent.

Cyclosporine is known to alter its own metabolism and toxicity by modulating the activity of selected P450 isoforms [13,14]. We previously reported that male rats

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administered cyclosporine for 28 days had a dramatic reduction in hepatic microsomal P450 3A2 and 2C11 protein expression and corresponding metabolic activity as compared with vehicle controls [13]. Female rats given cyclosporine had less of a reduction in kidney function as compared with males, despite female rats having cyclosporine trough blood levels nearly twice those of male counterparts. Furthermore, metabolic activity in female rats was not altered indicating that cyclosporine-mediated alterations in hepatic drug metabolism are gender-dependent and cyclosporine blood levels are not directly related to renal dysfunction in this model.

Inhibition of hepatic P450 is also known to be dependent on the length of dosing [15] and is apparently an indirect process, as in vitro addition of cyclosporine to hepatic microsomal protein does not inhibit microsomal metabolism [16]. Rats were given cyclosporine therapy for up to 28 days followed by 15 days of vehicle only administration. After 2 weeks, cyclosporine treated rats had significant reductions in hepatic P450 3A2 and 2C11 protein levels and in vitro metabolic activity. The greatest reductions were found after 28 days of dosing, with recovery to nearly predosing levels after drug removal and vehicle administration for 15 days. Thus, not only is cyclosporine-dependent suppression directly related to the length of treatment, but the suppression is reversible with the discontinuation of dosing.

The purpose of the study was to determine whether there was a dose-dependent suppression of hepatic P450 protein levels and metabolic activity during chronic cyclosporine administration or whether a threshold dose for suppression exists. We examined the modulation of microsomal oxidative metabolism and protein expression by cyclosporine following 2 weeks of oral dosing using escalating dose of the drug.

2. 2. Materials and methods

2.1. Animals

Thirty 10-week-old male Sprague–Dawley rats were purchased from Charles River Breeders (Wilmington, MA) and individually housed in wire bottom cages. Rats were kept in an animal care facility with a 12 h light/dark cycle and controlled temperature and humidity. Following a seven day acclimation period, rats were pair-fed a low sodium diet (17.4% protein, 64.7% carbohydrates, 5.2% fat, 0.05% sodium; Harlan Teklad, Madison, WI) and allowed access to tap water ad libitum. All procedures were approved by the Animal Care and Use Committee of The University of Texas at Austin prior to the start of the study. The Animal Care Department is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

2.2. Chemicals

Cyclosporine was provided in the oral solution dosage form (Sandimmune®; Novartis Pharmaceuticals, East Hanover, NJ), and diluted to the appropriate concentration with olive oil (Croda, Inc., Parsippany, NJ). Testosterone metabolites were purchased from Steraloids, Inc. (Wilton, NH). Acrylamide/bisacrylamide solution was purchased from National Diagnostics (Atlanta, GA). All other reagents used for gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). High-performance liquid chromatographic (HPLC) grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) in the highest purity available.

2.3. Drug treatment

Following 1 week of the low-salt diet to insure sodium depletion, rats were randomly assigned to five groups of six rats each. Rats were administered cyclosporine 5, 15, 30 or 50 mg/kg per day or olive oil vehicle via oral gavage for 14 days. Volumes of all doses were 1 ml/kg and were administered the same time each day to minimize chronobiological variability in cyclosporine toxicity [17]. The day after the final cyclosporine dose, rats were anesthetized with a single intramuscular injection of a mixture of ketamine 100 mg/ml, xylazine 20 mg/ml, acepromazine 10 mg/ml in a 1:1:1 ratio (v:v:v). Livers were isolated and immediately frozen in liquid nitrogen and stored at −80°C.

2.4. Microsome isolation

Microsomes were prepared from hepatic tissue using differential centrifugation [18]. In brief, one gram aliquots of liver tissue were homogenized in three volumes of Tris(hydroxymethyl)aminomethane (Tris) chloride buffer (pH 7.4), containing 150 mM potassium chloride and 1 mM ethylenediaminetetraacetic acid (EDTA), with a tissue homogenizer (Fisher Scientific, Pittsburgh, PA). Samples were then centrifuged at $9000 \times g$ for 20 min (GS-15R, F0630 rotor; Beckman Instruments, Inc., Palo Alto, CA). The supernatant was collected and centrifuged at $490\,000 \times g$ for 17 min (TL-100, TLA 100.4 rotor; Beckman Instruments, Inc., Palo Alto, CA). The supernatant was discarded and the pellet resuspended with a tissue grinder (Wheaton, Milleville, NJ) in a sodium pyrophosphate buffer, pH 7.4, containing 1 mM EDTA. The suspension was centrifuged again at $490\,000 \times g$ for 17 min. The supernatant was discarded and the washed pellet resuspended in Tris chloride buffer (pH 7.4), containing 20% glycerol. The samples were maintained at 4°C during the procedure and microsomes were stored at −80°C prior to analysis (within 1 week).

2.5. In vitro metabolism

An in vitro testosterone hydroxylation assay was used to determine hepatic microsomal P450-specific metabolic activity as previously outlined [13]. In brief, 1 ml reaction mixtures contained 0.1 M potassium phosphate (pH 7.4), 0.2 mg microsomal protein, 250 mM testosterone, in methanol and an NADPH regenerating system consisting of 0.5 mM β-nicotinamide adenine dinucleotide (NADP), 10 mM glucose-6-phosphate, 10 mM magnesium chloride, and 5 units glucose-6-phosphate dehydrogenase. After preincubation at 37°C for 3 min the reaction was initiated by the addition of glucose-6-phosphate dehydrogenase. Incubations proceeded for 15 min and were quenched with 5 ml dichloromethane and the internal standard (3.6 mmol 11α-hydroxyprogesterone) was added. The organic layer was removed and dried under a nitrogen stream. Dried extracts were dissolved in 200 μl methanol and stored at 4°C until analyzed (within 1 week).

2.6. Chromatography

Testosterone and metabolites were separated and quantified using HPLC. Twenty microliters of the in vitro metabolism extracts were injected into an HPLC system consisting of an automatic injector (SIL-10A) with dual solvent pumps (LC-10AS), and a variable wavelength UV-VIS-detector (SPD-10A). The system was operated by a system controller (SCL-10A) and a software package (CLASS-VP version 4.2; Shimadzu Scientific Instruments, Columbia). Metabolites were resolved at 40°C on a 150 × 4.6 mm C-18 column (Supelco, Bellefonte, PA). 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 20 min at 1 ml/min flow rate. A 20-min washout with 90% solvent A preceded each analysis. Absorbence of hydroxylated metabolites was monitored at 238 nm. Testosterone metabolites were quantified by comparison of peak area ratios (metabolite:internal standard) with those generated by authentic standards. Rates were determined under conditions that were linear with protein and time.

2.7. Western blot analysis

Microsomal protein concentrations were determined by the method of Lowry [19] using a commercially available assay kit (DC Protein Microassay; Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Proteins were separated using SDS-PAGE as described previously with an 8% polyacrylamide separating gel [20]. Prestained broad-range molecular weight markers (Bio-Rad Laboratories, Hercules, CA) were used for estimating protein molecular weight. Separated proteins was electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) using an electrophoretic blotter (Idea Scientific Co., Corvallis, OR) as outlined previously [21]. Prior to detection of P450 3A2 and 2C11 immunoreactive proteins, nitrocellulose sheets were blocked with 3% nonfat dry milk (NFDM) in Tris-buffered saline (TBS; 10 mM Tris–chloride buffer (pH 7.4), containing 0.9% sodium chloride). For the detection of P450 3A2 immunoreactive proteins, nitrocellulose sheets were blocked with 3% NFDM in TBS and incubated with 1:2,000 dilution of goat anti-rat 3A2 antibody (GenTest, Woburn, MA) in 3% NFDM in TBS and then a 1:2000 dilution of rabbit anti-goat horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) in 3% NFDM in TBS. For the detection of 2C11 immunoreactive proteins, blocked nitrocellulose sheets were incubated with a 1:2000 dilution of rabbit anti-rat 2C11 antibody (generously provided by Dr Edward T. Morgan, Emory University, Atlanta, GA) and then a goat anti-rabbit horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) in 3% NFDM in TBS. Immune complexes were detected by chemiluminescence with an ECL detection kit as described by the manufacturer (Amersham, Arlington Heights, IL) using Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY). Immunoreactive band density was measured using a flatbed scanner (LaCie, Beaverton, OR) and analyzed on a Power Macintosh 7200/90 computer using the public domain NIH Image program version 1.60 (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.8. Statistical analysis

Differences between rat groups were determined using one-way analysis of variance with Bonerroni–Dunn post-hoc analysis using the vehicle group as the control (SuperANOVA; Abacus Concepts, Berkeley, CA). Data is presented as mean ± standard error. Differences were considered significant when the probability of chance explaining the results was reduced to less than 5% ($P < 0.05$; $\alpha = 0.05$).

3. Results

3.1. Western blot analysis

In order to monitor the effect of cyclosporine dosing on specific P450 isoforms, immunoblot analysis was performed on hepatic microsomal protein. Microsomes from each rat showed a single immunoreactive band consistent with P450 3A2 whose intensity was inversely related to the dose of cyclosporine administered. Cyclosporine reduced P450 3A2 expression by 27% ($P = 0.047$) in rats given 5 mg/kg per day, by 34% ($P = 0.015$) with 15 mg/kg per day, by 65% ($P = 0.001$) with 30 mg/kg per day, and by 88% ($P < 0.001$) with a dose of 50 mg/kg per day as compared with control rats (Figs. 1 and 2).

When blots were probed for proteins immunoreactive to the 2C11 antibody, a single band was also found which was consistent with P450 2C11. Figs. 1 and 2 show that cyclo-

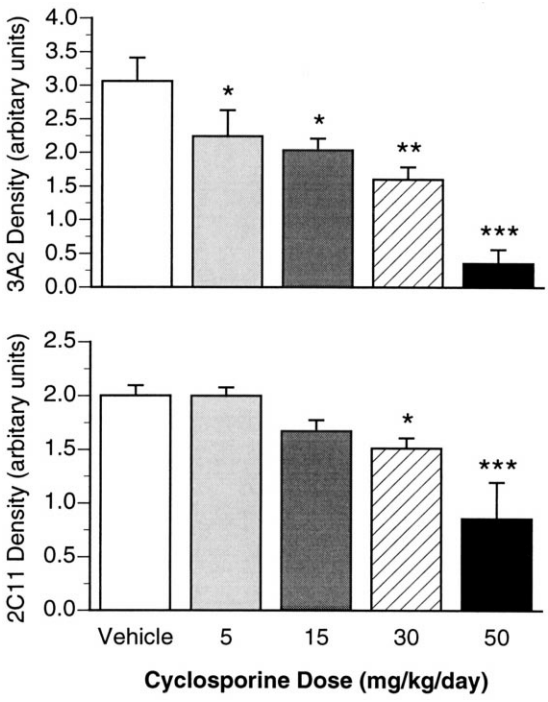

Fig. 1. Western blot analysis of hepatic P450 3A2 and 2C11 microsomal protein expression in rats given escalating doses of cyclosporine or drug vehicle for 14 days. $N = 6$ each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with vehicle controls.

sporine reduced the hepatic microsomal expression of P450 2C11 by 20% ($P = 0.062$) in rats receiving 15 mg/kg per day, by 25% ($P = 0.011$) with 30 mg/kg per day, and by 57% ($P < 0.001$) when given 50 mg/kg per day as compared with vehicle controls. In the group of rats dosed with cyclosporine 5 mg/kg per day, there was no reduction in the immunoreactive P450 2C11 protein ($P = 0.981$).

3.2. In vitro metabolism

The regio- and stereospecific hydroxylation of testosterone in vitro by microsomal proteins is an effective marker of specific P450 isoform activity. Similar to the results seen with the immunoblotting, cyclosporine treatment caused a dose-dependent suppression of P450 3A2 and 2C11 metabolic activity (Fig. 3). Compared with control rats, cyclosporine treatment reduced the in vitro production of the P450 3A2 predominant metabolite, 6β-hydroxytestosterone, by 17% ($P = 0.074$) with 5 mg/kg per day, by 27% ($P = 0.007$) with 15 mg/kg per day, by 40% ($P < 0.001$) with 30 mg/kg per day, and by 81% ($P < 0.001$) when the dose was 50 mg/kg per day.

Similar results were found with the in vitro production of the P450 2C11 predominant testosterone metabolite, 2α-hydroxytestosterone. Cyclosporine reduced microsomal 2α-hydroxytestosterone production by 5% ($P = 0.571$) at a dose of 5 mg/kg per day, by 19% ($P = 0.034$) with 15 mg/kg per day, by 50% ($P < 0.001$) with 30 mg/kg per day,

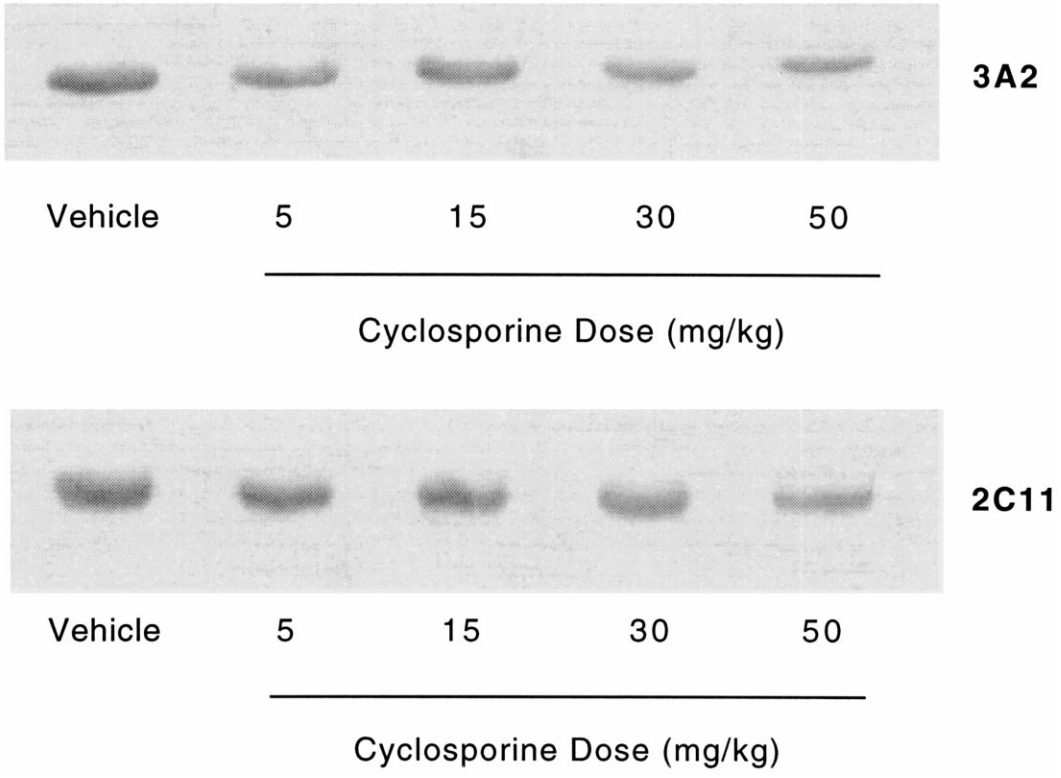

Fig. 2. Representative Western blots of P450 3A2 and 2C11 immunoreactive proteins from hepatic microsomes isolated from rats given drug vehicle or escalating doses of cyclosporine for 14 days.

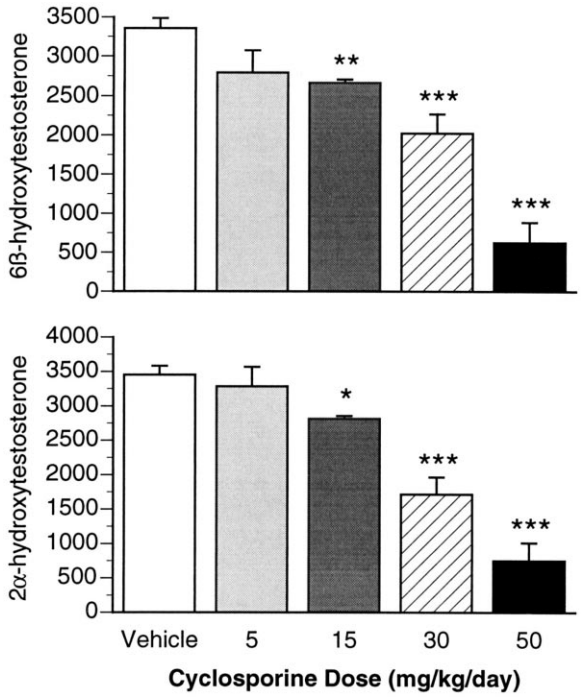

Fig. 3. In vitro testosterone hydroxylation by hepatic microsomes following 14 days of oral cyclosporine administration or vehicle. Units of metabolite production are pmol metabolite/min/mg microsomal protein. $N = 6$ each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with vehicle controls.

and by 78% ($P < 0.001$) when given 50 mg/kg per day as compared with the control group.

In order to determine whether or not the decrease in hepatic microsomal activity was specific to P450 3A2 or 2C11 or generalized metabolic suppression, we examined the in vitro production of two additional testosterone metabolites which have not yet been reported to be modulated by cyclosporine treatment. We found that cyclosporine did not significantly alter the in vitro hepatic microsomal production of 7α-hydroxytestosterone, a marker of P450 2A1 activity, or 16β-hydroxytestosterone, a marker of P450 2B1 metabolic activity (Fig. 4).

4. Discussion

A clear association between cyclosporine dosing and markers of effect or toxicity does not currently exist in the transplant patient population. The cause of this lack of association is multifactorial, and includes concomitant medications and changes in organ function over time. Even with chronic cyclosporine administration, transplanted patients have an increase in steady-state blood levels which masks the relationship between drug dosing and circulating cyclosporine levels. While this increase has been attributed to enhanced oral bioavailability with chronic dosing, the direct effect of cyclosporine administration on intestinal or hepatic P450-mediated metabolism has been given little attention. In the present study, we investigated the effect of increasing oral doses of cyclosporine on hepatic P450-mediated in vitro metabolism in a rat model of chronic cyclosporine nephropathy. We found that cyclosporine selectively suppressed hepatic microsomal P450 protein levels and catalytic activity which was directly dependent on the dose administered. Rats receiving daily oral cyclosporine doses 15, 30 and 50 mg/kg showed significant hepatic P450 suppression as compared with rats given vehicle only over a 2 week period.

We have previously reported that chronic cyclosporine dosing causes a gender-specific reduction in P450-mediated hepatic metabolism [13]. Male rats administered cyclosporine for 4 weeks had a marked reduction in P450-mediated drug metabolism resulting in circulating blood levels of cyclosporine much greater than female counterparts. Thus, male rats would require less drug to be administered to achieve the same therapeutic levels. The mechanism of this reduction remains unclear but may be caused by hormonal differences between male and female rats [22,23]. Specifically, the secretion pattern of growth hormone in adult rats regulates gender-dependent P450 isoforms, such as P450 3A2, 2C11, and 2C12. In male rats, growth hormone is secreted in a low-frequency, high-amplitude manner while in female rats, secretion is in a high-frequency, low-amplitude pattern. Disruption of this secretion pattern in male rats results in a 'feminization' of the hepatic expression of P450 enzymes. Indeed, male rats given cyclosporine for 1 month had a dramatic reduction in both protein levels and meta-

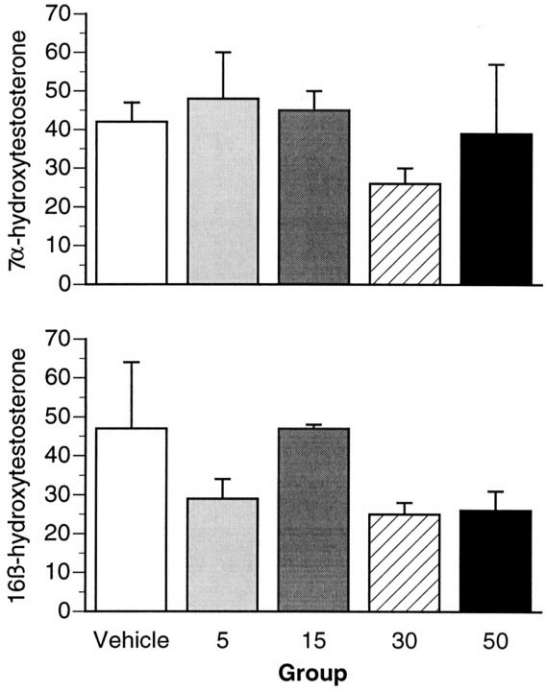

Fig. 4. In vitro production of testosterone metabolites by hepatic microsomes isolated from rats given cyclosporine or vehicle for 14 days. Units of metabolite production are pmol metabolite/min/mg microsomal protein. $N = 6$ each group.

bolic activity of the male-specific P450 isoforms, 3A2 and 2C11. Furthermore, there was a significant increase in female-specific hepatic microsomal 5α;-reductase activity as compared with controls [22,23].

Not only is cyclosporine-induced suppression of hepatic P450 gender-dependent, it is also directly related to the length of drug administration [15]. Rats given cyclosporine for up to 28 days had a time-dependent suppression of cyclosporine metabolism which became significant following the first 2 weeks of treatment. Circulating cyclosporine blood levels were markedly elevated at 14 days and remained so until at least 1 week after cessation of drug dosing. This elevation in cyclosporine levels occurred in conjunction with the suppression of hepatic microsomal P450 3A2 protein levels and in vitro metabolic activity.

In the present study, we investigated the role of cyclosporine dosing level on the regulation of P450-mediated drug metabolism. Based on our previous findings, we administered cyclosporine at escalating doses over a 2-week period in order to determine whether or not higher doses of cyclosporine would result in greater hepatic P450 suppression without causing complete repression of P450 3A2 or 2C11 protein levels. We found that hepatic levels of P450 3A2 proteins were significantly reduced in all rats receiving cyclosporine as compared with vehicle controls (Figs. 1 and 2). In contrast, hepatic P450 2C11 levels were also reduced, but only to a significant extent in rats administered cyclosporine 30 and 50 mg/kg per day.

Metabolic activity of hepatic microsomal P450 was also reduced by cyclosporine dosing (Fig. 3). The in vitro production of 6β-hydroxytestosterone by hepatic microsomes is a marker of P450 3A2 metabolic activity and can be used as a surrogate measure of in vivo drug metabolism. Following 14 days of cyclosporine administration, production of 6β-hydroxytestosterone by liver microsomes was markedly reduced in rats in the 15, 30 and 50 mg/kg per day dosing groups. These results support the Western blot data which showed a corresponding decrease in immunoreactive P450 3A2 protein levels. Analogous to the production of 6β-hydroxytestosterone, 2α-hydroxytestosterone is a marker of P450 2C11 metabolic activity. Rats given cyclosporine 15, 30 and 50 mg/kg per day also had a significant reduction in 2α-hydroxytestosterone production which paralleled the decrease in P450 2C11 immunoreactive proteins. Thus the parallel effect of cyclosporine dosing on hepatic P450 protein levels and activity was a reduction in gene expression or RNA translation and not a stabilization of an inactive form of the protein as has been reported for other P450 3A substrates, such as erythromycin [24].

In order to determine whether or not the reduction of hepatic P450 protein and activity was selective for certain isoforms or represented a generalized suppression, we measured the activity of P450 isoforms not believed to be gender-specific or altered by chronic cyclosporine therapy. We examined the in vitro microsomal production of 7α-hydroxytestosterone and 16β-hydoxytestosterone, which are markers of P450 2A1 and 2B1 activity in the rat, respectively [25,26]. Cyclosporine administration did not have any discernable effects on the metabolic activity of P450 2A1 or 2B1 regardless of the dose administered (Fig. 4). Thus, while cyclosporine does suppress P450 protein expression and catalytic activity, the effect is specific for certain P450 isoforms and does not represent an overt hepatotoxicity or widespread suppression.

A threshold for the suppression of P450 protein expression and catalytic activity appears to be present using this rat model. Even the lowest dose of cyclosporine administered caused a significant reduction in P450 3A2 protein expression. A significant reduction in catalytic activity of P450 3A2, however, required a much higher dose of cyclosporine at 15 mg/kg per day. The repression of hepatic P450 2C11 protein expression was more resistant to the effect of cyclosporine dosing in that a minimum of 30 mg/kg per day was required to demonstrate a significant change. In contrast, a lower dose of 15 mg/kg per day was required to produce a significant decrease in P450 2C11 catalytic activity. Thus, the threshold for dosing in this model is dependent on the specific P450 protein and whether or not the measurement is protein expression or catalytic activity.

In conclusion, cyclosporine is an immunosuppressant widely used as prophylaxis against allograft rejection in transplant patients. In rats, cyclosporine is known to suppress its own metabolism in a gender-dependent manner and based on the length of therapy. Data from the present study show that the suppression of hepatic P450 3A2 and 2C11 mediated xenobiotic metabolism is dependent on the dose administered, with higher doses causing the greatest metabolic suppression. The mechanism of the suppression remains unclear, but likely is related to endocrine function. Further studies are warranted to investigate the role of the endocrine system and cyclosporine-associated changes in drug metabolism in both rodents and humans.

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