Metabolism of cyclosporine by cytochromes P450 3A9 and 3A4

P.A. KELLY¹,³, H. WANG², K.L. NAPOLI¹, B.D. KAHAN¹ and H.W. STROBEL²

¹Division of Immunology and Organ Transplantation, ²Department of Biochemistry and Molecular Biology, University of Texas-Houston Health Science Center, and ³College of Pharmacy, University of Houston, Houston, Texas, USA

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SUMMARY

The ability of P450 3A9 to transform cyclosporine was studied and compared to that of human P450 3A4. Purified P450 3A4 and P450 3A9 proteins were reconstituted in a system containing potassium phosphate buffer, lipids, NADPH–P450 reductase, and glutathione with NADPH added to initiate the reaction. Cyclosporine was added alone and with or without the inhibitors, ketoconazole or troleandomycin. High performance liquid chromatography with ultraviolet (HPLC/UV) techniques were used to analyze for cyclosporine metabolites. Both P450 3A4 and P450 3A9 transformed cyclosporine to three metabolites: AMI, AM9, and AM4n. P450 3A4 predominantly formed AMI (63% of metabolites formed) while P450 3A9 formed AM4n (59% of metabolites formed). Ketoconazole (0.5 μM) completely inhibited P450 3A9 catalyzed formation of AMI and AM9 and reduced AM4n formation to 28% of control. AM4n, AM1, and AM9 formation catalyzed by P450 3A4 was reduced to 50%, 30%, and 10% of control, respectively, by 0.5 μM ketoconazole. Troleandomycin (> 10 μM) inhibited the formation of AM4n by P450 3A4 and P450 3A9 to 60–70% of control, while the production of AM1 by P450 3A4 was increased to 120% of control and the production of AM1 by P450 3A9 was inhibited to 50% of control. Inhibition of P450 3A4 by troleandomycin (> 10 μM) reduced the formation of AM9 to 40% of control, but only reduced P450 3A9 formation of AM9 to 80% of control. This study shows that rat P450 3A9 is capable of transforming cyclosporine to multiple metabolites similar to those generated by human P450 3A4.

INTRODUCTION

Cyclosporine (CsA) is an immunosuppressive agent currently used clinically for prophylaxis against rejection in solid organ transplantation. Extensive hepatic metabolism of CsA results in three primary metabolites: the hydroxylated metabolites, AM1 and AM9; and the N-demethylated metabolite AM4n (1,2). Cytochromes P450 3A are thought to be predominantly, if not exclusively, responsible for the metabolism of CsA (3,4). In untreated adult female rats, hepatic levels of P450 3A1 and 3A2 are not detectable by assessment of specific monohydroxyandrostenedione metabolite formation or by immunoquantitation in isolated microsomes (5). Female rats are,
however, capable of metabolizing CsA (6,7). Evidence for involvement of an unknown 3A isozyme or an enzyme other than the CYP3As that is capable of metabolizing CsA was provided by Prueksaritanont and colleagues, who demonstrated that enzyme(s) other than rat 3A1 and 3A2 play a role in the metabolism of CsA in female rats (6). This unidentified enzyme was found to be female-specific and inducible by ethinyl estradiol but not by dexamethasone (6). The expression levels of rat CYP3A1/2 and human P450 3A4 are reduced by estrogen and induced by dexamethasone (9-11).

Recently, Wang and Strobel have documented the existence of a new female-specific cytochrome P450 3A, P450 3A9, which has been shown to be active in N-demethylation of erythromycin, imipramine, benzphetamine, and ethylmorphine, and was active in both 2- and 4-hydroxylation of 17β-estradiol in a reconstituted system (8). Interestingly, the sexually dimorphic expression of this novel form was found to be estrogen dependent and was inducible solely by estrogen, but not by dexamethasone (8). This may suggest that P450 3A9 is one of the major enzymes responsible for CsA metabolism in female rats. Therefore, in this study, we examined the ability of purified P450 3A9 to metabolize CsA in a reconstituted system and compared this activity to that of the purified human P450 3A4. We also examined the effects of two cytochrome P450 inhibitors, ketoconazole and troleandomycin, on the metabolism of CsA by these two enzymes. Our results show that P450 3A9 is capable of transforming CsA to the same three major metabolites as those catalyzed by human P450 3A4, suggesting a role for 3A9 in the metabolism of CsA in female rats.

MATERIALS AND METHODS

Recombinant P450 3A9 protein was expressed in *Escherichia coli* using the pCWOri+ expression vector and purified using methods previously described (8). Purified P450 3A4 was purchased from Panvera Corp. (Madison, WI, USA). NADPH–P450 reductase was purified from rat liver microsomes using the methods described previously (12,13). CsA (Sandoz, East Hanover, NJ, USA), ketoconazole (KETO, Gentest Corp., Woburn, MA, USA), and troleandomycin (TAO, Sigma, St Louis, MO, USA) were dissolved and further diluted in methanol.

Reconstitution conditions for the P450 3A enzymes required a 1:1:1 lipid mixture of L-α-dilauroyl phosphatidylcholine, L-α-dioleoyl phosphatidylcholine, and phosphatidylserine. Assays were performed in a final volume of 1 ml with 100 mM potassium phosphate buffer (pH 7.25), containing 100 pmole P450 3A9 protein or 50 pmole P450 3A4 protein, saturating NADPH–P450 reductase (0.5 units), 20 μg lipid mixture, 3.0 mM glutathione, and CsA as substrate, with or without inhibitors. Inhibitors were added at the same time as substrate to approximate more closely inhibition that would be observed in an in vivo setting. The reaction mixture was pre-incubated at 37°C for 5 min and the reaction was initiated by the addition of 0.5 mM NADPH and incubated at 37°C for 1 h. CsA metabolism by purified P450 3A9 was found to be linear during the 60 min incubation. Linearity of metabolism of CsA by human cytochromes P450 has been shown previously using both human liver microsomes as well as human liver, kidney, and intestine slices (14,15). Each reaction was terminated at the end of the incubation by the addition of 1 ml acetonitrile and chilling in an ice bath. Samples were initially partially dried down under a constant stream of nitrogen to remove the acetonitrile, then 1 ml water and 10 ml ethyl ether were added. After shaking and centrifugation, the organic layer was transferred to a clean tube and was dried down under a constant stream of nitrogen. The samples were then dissolved in mobile phase (acetonitrile:methanol:water, 47:23:30) and transferred to HPLC vials for injection onto the HPLC instrument.

The metabolites of CsA were separated by HPLC equipped a Waters 710B autosampler (Waters Corp., Milford, MA, USA) and Waters 510 pump (flow rate 1 ml/min). Separation and detection of CsA metabolites and parent drug was performed using tandem Supelcosil LC-18 columns (Supelco, Bellefonte, PA, USA; 30 cm x 4.6 mm) heated to 75°C and a Waters 486 UV detector (wavelength 210 nm). Waters chromatography software (Millennium v. 2.1) was utilized for the chromatographic analysis of the samples. Metabolite profiles generated were compared to profiles of 7 authentic CsA metabolites. The CsA metabolites were kindly supplied by Dr Randall Yatscoff, Edmonton, Alberta, Canada.

RESULTS

Transformation of cyclosporine

Both P450 3A4 and P450 3A9 transformed CsA to three principal metabolites, namely, the hydroxylated forms AM1 and AM9 and the demethylated form AM4n. Representative chromatograms of CsA metabolites catalyzed by P450 3A9 are shown in Figure 1. P450 3A4 predominantly formed AM1, with AM9 and AM4n being