ETHYNYLESTRADIOL-MEDIATED INDUCTION OF HEPATIC CYP3A9 IN FEMALE RATS: IMPLICATION FOR CYCLOSPORINE METABOLISM

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ABSTRACT:

Repeated treatment of female rats with the synthetic estrogen ethynylestradiol (EE₂) increases the formation of the cyclosporine A (CyA) metabolites AM1c and AM9 by 3-fold, whereas the formation of AM1 and AM4N is not significantly enhanced. The formation of all four CyA metabolites was inhibited by greater than 80% by the CYP3A-selective substrate midazolam or polycylic anti-rat CYP3A IgGs in liver microsomes of untreated and EE₂-induced rats. In contrast, anti-rat CYP2C6 IgGs had little effect, indicating the involvement of a CYP3A but not 2C6 in this EE₂-stimulated CyA metabolism. Semi-quantitative reverse-transcriptase polymerase chain reaction was used to determine the mRNA content for four CYP3A genes (CYP3A2, CYP3A9, CYP3A18, and CYP3A23) in livers of control and EE₂-treated female rats. EE₂ selectively induced CYP3A9 by 3.3-fold whereas the expression of CYP3A18 and CYP3A23 was slightly decreased; neither CYP3A2 mRNA nor CYP3A1 mRNA was detectable in these EE₂-treated livers. To determine whether rat liver microsomal CYP3A9 was indeed responsible for the EE₂-stimulated CyA metabolism, a recombinant CYP3A9 was heterologously expressed in Escherichia coli. When functionally reconstituted, this enzyme was active in metabolizing CyA preferentially to its AM9 and AM1c metabolites as compared with CYP3A4. These results thus support the notion that the increased CyA-metabolizing capacity of EE₂-treated female rat liver microsomes is due to the induction of the CYP3A9 enzyme.

Cyclosporine (CyA)¹ is a cyclic undecapeptide used in transplantation medicine and to treat autoimmune diseases. It undergoes extensive hepatic and intestinal metabolism, resulting in three primary metabolites, two hydroxylated products, AM1c and AM9, and one N-demethylated metabolite, AM4N. A cyclic derivative, AM1c, of AM1 is also formed (Maurer et al., 1984). To date, cytochromes CYP3A have been identified as the major CyA-metabolizing enzymes in human and rabbit livers (Bertault-Peres et al., 1987; Kronbach et al., 1989). Repeated treatment of male and female rats with ethynylestradiol (EE₂), a cytochrome P-450 (P-450) suicide inactivator both in vivo and in vitro (White and Muller-Eberhard, 1977; Pichard et al., 1989), increases CyA metabolism, although it decreases CYP3A-dependent testosterone 6β-hydroxylase activity. Our previous studies (Prueksaritanont et al., 1993) of immunoblot analysis of liver microsomes from EE₂-treated female rats using specific monoclonal antibodies raised against purified rat CYPs 3A1 and 3A2, and polyclonal antibodies to CYPs 2C6, 2C7, 2C11, and 2E1, revealed an increase of CYP2C6 and no detectable levels of CYPs 3A1 and 3A2. EE₂ treatment also enhanced progestrone 21- and (S)-warfarin 7-hydroxylases (both selective functional markers for 2C6) 2- to 3-fold, whereas testosterone 6β-hydroxylase was markedly decreased, thereby excluding the contribution of CYPs 3A1 and 3A2 to the increased CyA metabolism (Prueksaritanont et al., 1993)).

Recently, the cDNAs of five members of the rat CYP3A subfamily have been characterized, namely CYP3A1 (Gonzalez et al., 1985), CYP3A2 (Gonzalez et al., 1986), CYP3A9 (Wang et al., 1996; Mahnke et al., 1997; Wang and Strobel, 1997), CYP3A18 (Strotkamp et al., 1995; Nagata et al., 1996; Robertson et al., 1998), and CYP3A23 (Kiriti and Matsubara, 1993; Komori and Oda, 1994). However, no polymerase chain reaction (PCR) product for CYP3A1 was detected using the specific primer pairs in male and female rat livers (Mahnke et al., 1997). This was also confirmed by Northern blot analyses using two specific oligonucleotides designed to distinguish CYP3A23 and CYP3A1 mRNAs, which revealed signal intensity only corresponding to hepatic CYP3A23 mRNA in dexamethasone (DX)- and pregnenolone 16α-carbonitrile-treated Sprague-Dawley rats. CYP3A1 may be therefore an allelic variant of CYP3A23. To investigate whether the enzyme(s) responsible for the increased CyA metabolism after EE treatment belongs to the CYP2C or CYP3A sub-
family, inhibition experiments with specific anti-rat CYPIgGs and a relatively selective competitive CYP3A substrate were performed. Furthermore, semiquantitative reverse transcriptase (RT)-PCR was used to measure the levels of CYP3A2, CYP3A9, CYP3A18, and CYP3A23 expression in livers of control and EE2-treated female rats using β-actin and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as internal standards. The possible role of these P-450s in CyA metabolism is discussed, and evidence is provided to support the notion that EE2-induced CYP3A9 is the enzyme responsible for this metabolism.

Experimental Procedures

Materials. CyA, AM1, AM1c, AM9, and AM4N were supplied by Sandoz Ltd. (Basel, Switzerland). Midazolam and 1-OH-midazolam were a generous gift from Hoffmann-La Roche (Basel, Switzerland). EE2, and the constituents for the NADPH-generating systems were purchased from Sigma Chemical Co. (St. Louis, MO) and used without additional purification. [14C]progesterone was obtained from New England Nuclear (Boston, MA). 21-Hydroxyprogesterone was purchased from Steraloids (Wilton, NH). Tris-saturated phenol, chloroform, isopropanol, and ethanol were obtained from Baker (Deventer, the Netherlands). Moloney murine leukemia virus reverse transcriptase, RNase block ribonuclease inhibitor, and random primers were obtained from Stratagene (Heidelberg, Germany). Sephadex G-75 and 2′-deoxyoctonucleotide 5′-triphosphate (dNTP set) were purchased from Pharmacia (Uppsala, Sweden) and Taq DNA polymerase was obtained from Perkin Elmer (Madison, WI). All other chemicals were obtained as stated (Wang et al., 1998).

Immunoochemical Procedures. Polyclonal antibodies were raised in rabbits against purified liver P-450s 2C6 and 3A as described previously (Bornheim and Correia, 1989a). IgGs were purified from rabbit sera after application to a High Trap Protein A column (5 ml; Pharmacia, Piscatway, NJ), equilibrated with Tris/HCl (0.05 M, pH 8.0). After washing with 5 column-volumes of the same buffer, the protein was eluted with 0.1 M glycine/HCl (pH 2.75). To stabilize the IgGs, the pH of the fractions was immediately adjusted to pH 7.4 with 1 M Tris-HCl. The specificity of both IgGs was validated with known functional markers of CYPs 2C6/7 and CYP3A. Polyclonal anti-rat CYP3A IgGs (10 mg IgG/ml; P-450) were shown to inhibit 1-OH-midazolam formation by greater than 85%. Liver microsomes (1 nmol P-450) from EE2-pretreated female rats and midazolam (50 μM) were incubated at 37°C for 20 min in the presence of an NADPH-generating system (final volume, 1 ml) and the relative formation of 1-OH-midazolam was assessed by HPLC as described (Gascon and Dayer, 1991). Progestrone-21-hydroxylase activity, a CYP2C-selective marker, was inhibited by anti-rat CYP2C6 IgGs (10 μg IgG/ml; P-450) by greater than 91%. Liver microsomes (1 nmol P-450) from EE2-pretreated female rats and progesterone (250 nmol) containing [14C]progesterone (1 μCi) were incubated at 37°C for 10 min, in the presence of an NADPH-generating system in a final volume of 1 ml. The reaction was terminated with ethyl acetate (2 × 3 ml). Nonlabeled 21-OH-progesterone was then added, the ethyl acetate layers collected, dried under a stream of nitrogen, and dissolved in 0.2 ml (43% methanol, 1.1% acetonitrile). This solution (100 μl) was HPLC-assayed as described (Pruesskaranont et al., 1993). 21-OH-progesterone was identified by its coelution with a known standard, collected, and quantified by liquid scintillation counting. Parallel incubations with preimmune sera were run as controls.

Animals and Microsomal Preparation. Female Sprague-Dawley rats (220–250 g) were obtained from the Institut für Versuchstieranstalt und-haltung, University of Vienna, Himberg, Austria. The rats were divided into three groups: 1) control (vehicle only s.c.) for 5 days; 2) EE2 (5 mg/kg/day s.c.) for 5 days; and 3) DX (100 mg/kg/day i.p.) for 5 days. EE2 and DX solutions for injection were prepared in propylene glycol and vegetable oil at concentrations of 3 and 25 mg/ml, respectively. The animals were treated daily between 9:30 and 10:30 AM and sacrificed by decapitation 24 h after the last injection. Livers were homogenized in 0.1 M phosphate buffer (pH 7.4), and liver microsomes were prepared using standard procedures (Bornheim and Correia, 1989b). P-450 content was determined by the method of Omura and Sato (1964), using a Uvikon 941 (Kontron Instruments, Neufarn, Germany) UV-visible spectrophotometer. Protein concentration was determined by the method of Lowry et al. (1951), using BSA as the standard. Microsomes were used in the incubation studies on the day of the preparation.

CyA Metabolism by Liver Microsomes. Microsomes (250 pmol P-450), NADPH (1 mM), isotonic acid (5 mM), and isotonic acid dehydrogenase (0.5 U/ml) were preincubated at 37°C for 5 min in 0.05 M phosphate buffer, pH 7.4 (final volume, 1.0 ml). The reaction was started by the addition of CyA (final concentration, 30 μM) in dimethyl sulfoxide (DMSO) and its metabolism was assessed by the formation of AM1, AM9, AM1c, and AM4N after a 30-min incubation at 37°C. Extraction and HPLC analysis were performed with minor modifications as described previously (Pruesskaranont et al., 1993). Briefly, after the reaction was stopped by the addition of a mixture of acetatinol/methanol/zinc sulfate (35:60:5, v/v/w; 3 ml), dihydrocyclosporine (1 μM) was added as an internal standard. After centrifugation, the supernatant was removed and further extracted with hexane (3 ml). The hexane layer was discarded, and the aqueous layer was passed through a C18-Bond Elut column (VARIANT, Harbor City, CA), equilibrated with 5 ml of methanol and water, respectively. The column was washed with acetonitrile in water (35% v/v; 2 ml), and CyA and its metabolites were eluted with acetonitrile (100%; 2 ml) and dried under a stream of nitrogen. The residue was reconstituted with 200 μl of the mobile phase before injection (150 μl) on the HPLC column. Chromatographic separations were performed on a C18-ultrasphere reversed phase column (5 μm, 250 × 4.6 mm; Beckman, Deerfield, IL) preceded by a 10-μm reversed phase (C18) guard column and a 2-μm in-line filter (Alltech) at a column temperature of 70°C, using a mobile phase of acetonitrile/methanol/water/phosphoric acid (55:10.35:5; v/v), pH 3.

Chemical Inhibition of CyA Metabolite Formation. Midazolam (10 μl of a 0-, 0.5-, 1-, 2.5-, 5-, 10-, or 20-mM solution in ethanol) was added to 1.0 mg of microsomal protein on ice. Reaction mixtures (1.0 ml; n = 3) were preincubated in a shaking water bath at 37°C for 3 min in the presence of an NADPH-generating system (1 mM NADPH, 5 mM isotonic acid, 0.5 U/ml isotonic acid dehydrogenase). Each reaction was initiated by the addition of CyA in DMSO (final concentration 30 μM) at t = 0 min. Control experiments included the same solvent without inhibitor. Ethanol concentrations ≤5% (v/v) did not affect the rates of CyA metabolism formation. After 30 min at 37°C, reactions were stopped and analyzed by HPLC as described above. To characterize the nature of the inhibition, graphical analyses with Lineweaver-Burk and Dixon plots were performed. In these experiments, midazolam and CyA were used in final concentrations ranging from 0 to 100 and 0.2 to 2 μM, respectively, in incubations with control female liver microsomes, and AM9 formation was assayed exactly as described above.

Immunoinhibition of CyA Metabolism. Premimmune sera or rabbit polyclonal anti-rat CYP2C6 or 3A IgG preparations, in amounts shown, were mixed with undiluted control and EE2-treated rat liver microsomes (containing 500 pmol of total P-450). The reaction tubes were gently mixed and allowed to stand for 30 min at room temperature. An NADPH-generating system was then added, and the incubation volume was adjusted to 1.0 ml with 0.05 M phosphate buffer, pH 7.4. The samples were preincubated at 37°C for 5 min. At t = 0 min, CyA in DMSO (final concentration, 30 μM) was added, and the samples were incubated at 37°C for 30 min and analyzed as described above.

RNA Extraction and cDNA Synthesis. Total RNA was obtained from frozen rat liver (0.5–1 g) according to Chomczynski and Sacchi (1987). The RNA content was determined spectrophotometrically at 260 nm, and 5 μg of total RNA was taken for reverse transcription. Random primer (300 ng) was added and primer annealing was performed by incubation for 10 min at 55°C and 10-min progressive cooling at room temperature. dNTP (4 mM each), 5 μl of reaction buffer (Stratagene), 40 U of RNase block ribonuclease inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase were added to a final volume of 50 μl, and samples were incubated at 37°C for 1 h. The reaction was stopped by heating to 90°C for 5 min. Control samples contained distilled water instead of RNA.

PCR. Two and a half microliters of reverse transcription sample was added to the corresponding PCR mixture containing 0.5 μM sense and antisense primer, 2 mM MgCl2, 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, and 80 μM dNTPs. After heating at 95°C for 5 min and cooling to the primer-annealing temperature for 5 min (60°C), 2 U of Taq DNA polymerase was added. The primers and the size of the PCR products are listed in Table 1. Amplification conditions for all primer pairs were 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C with a final incubation of 10 min at 72°C (Tröblik-
Thermostorer; Biometra, Göttingen, Germany). The number of cycles ranged from 28 to 33 depending on signal intensity and was well below the plateau phase of amplification for all target sequences in this system. For every series of experiments, two negative controls containing no cDNA or no reverse transcriptase were added. Complification in the same tube was performed with primer pairs for CYP3A9 or CYP3A23 and β-actin. For all other CYP3A genes, a β-actin segment was amplified separately and used as an external standard, because interaction of primer pairs caused a decrease in signal intensity. In addition to β-actin, GAPDH was also used as an external standard in the PCR experiments.

Quantification of cDNA Segments. Ten microliters of the PCR samples were electrophoresed in agarose (2%) and then stained with ethidium bromide. Five micrograms of HincII- or HaelIII-digested αX-174 RF DNA (Pharmacia) was used for molecular weight determinations and quantitative standardization. The band intensities were measured using a CCD camera (Cybertech, Berlin, Germany) and quantified using a two-dimensional analyzing software (Cybertech). Signal intensity ratios of the CYP3A and β-actin PCR products were expressed as molar ratios after correction for the different segment lengths.

Expression of CYP3A9 and CYP3A4. A full-length CYP3A9 cDNA was cloned into pET-15b plasmid vector (Novagen; carrying an N-terminal Hisb-Tag) between the Xhol (5′) and Bpu 1102I (3′) (Mahnke et al., 1997) and expressed in E. coli AD494 (DE3) at 30°C, in the presence of δ-aminolevulinic acid (75 mg/liter) with IPTG (1 mM) induction, exactly as described (Wang et al., 1998). Bacterial cells were harvested at 48 h, when maximal CYP3A9 expression yields (A 200 nm) were obtained. The cells were lysed by subjection to a French Press and sonication. Cell membranes were prepared and solubilized with CHAPS in the presence of protease inhibitors, as described (Wang et al., 1998). In parallel, cell membranes containing expressed CYP3A4 were prepared exactly as described before (Wang et al., 1998), and served as the corresponding positive controls.

Functional Assays for of CYP3A9 and CYP3A4. To assay testosterone 6β-hydroxylase activity, solubilized membranes containing either CYP3A9 (50 pmol) or CYP3A4 (50 pmol) were functionally reconstituted with NADPH-P-450 reductase and cytochrome b5 in molar ratios of 1:4:2, respectively, and incubated at 37°C for 10 min with testosterone (0.5 mM) as the substrate, as described (Wang et al., 1998). The testosterone metabolites were extracted and the relative amounts of 6β-hydroxytestosterone formed in the incubation mixtures were analyzed by HPLC with UV monitoring at 240 nm, with an authentic sample of 6β-hydroxytestosterone as a standard. CyA metabolism was assayed in a final volume of 1 ml with 100 mM potassium phosphate buffer, pH 7.4, containing either CYP3A9 (50 pmol) or CYP3A4 (50 pmol), NADPH-P-450 reductase, and cytochrome b5, in molar ratios of 1:4:2, respectively, 20 μl Lubrol (0.2%), lipid mixture (150 μg), GSH (3.0 mM), NADPH (1 mM), isocitric acid (5 mM), and isocitric acid dehydrogenase (0.5 U/ml). The lipid mixture consisted of a 1:1:1 mixture of 1-α-dilauroyl phosphatidylcholine, phosphatidylserine, and 1-α-dioleoyl phosphatidylcholine. The reaction was started by the addition of CyA (final concentration, 30 μM) in DMSO and the formation of AM1, AM9, AM1c, and AM4N metabolites monitored after 30 min of incubation at 37°C, as described above.

Data Analyses. For characterization of AM9 formation, CyA concentrations were varied up to 30 μM and kinetic parameters were estimated by nonlinear regression analysis (Prism 3.0; GraphPad Software Inc., San Diego, CA). Midazolam inhibition was characterized by graphical analyses with Lineweaver-Burk and Dixon plots. If not otherwise indicated, values are expressed as mean ± S.D. of three individual experiments. Statistical differences from control values were evaluated using Student’s paired t test, at a significance level of p < .05.

Results

CyA Metabolism by Liver Microsomes. Table 2 shows the formation of three primary CyA metabolites (AM1, AM9, and AM4N) and one secondary metabolite (AM1c) by liver microsomes from control, EE2-, and DX-pretreated female rats. Treatment of female rats with the CYP3A3a inactivator EE2 increased the hepatic microsomal formation of AM9 and AMic by greater than 2.7- and 3.2-fold, respectively, whereas the corresponding formation of AM1 and AM4N was only slightly but nonsignificantly enhanced by 23 and 37% in confirmation of previous findings (Prueksaritanont et al., 1993). On the other hand, liver microsomes from rats pretreated with DX, a CYP3A inducer, were highly active in metabolizing CyA with corresponding significant increases of 9.2-, 5.2-, 3.4-, and 3.7-fold, respectively, over the control liver microsomal values. DX did not further induce AM1c formation over that of EE2 pretreatment. Total P-450 content was not affected by EE2 treatment (0.61 ± 0.11 and 0.56 ± 0.09 nmol/mg protein, in EE2-treated and control rat livers, respectively), but increased markedly after treatment with DEX (1.27 ± 0.14 nmol/mg protein). Figure 1 depicts representative Michaelis-Menten plots for AM9 formation by liver microsomes from control (Fig. 1A) and EE2-treated (Fig. 1B) female rats. The apparent K_M value for AM9 formation in EE2-pretreated female rats was slightly increased but not significantly different from the values found in control female rats (1.44 ± 0.26 and 1.02 ± 0.19 μM, respectively), whereas the V_max value was approximately 2.5-fold higher after EE2 treatment (8.76 ± 1.55 and 2.93 ± 0.71 pmol/mg protein/min).

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Eadie-Hofstee analyses of these data revealed no biphasic kinetics at CyA concentrations up to 30 μM.

Chemical Inhibition of CyA Metabolism. A number of chemicals are known to selectively inhibit certain P-450 enzymes (Halpert et al., 1994; Wandel et al., 1998). Preincubation of liver microsomes with midaizolam (25–200 μM), a CYP3A3a substrate, resulted in a decrease of CyA metabolism at concentrations >25 μM. Specifically, midaizolam (200 μM) decreased the formation of AM1, AM9, and AM4N by control microsomes by 86, 91, and 85%, respectively (Fig. 2A). Because of the negligible AM1c formation by control rat liver microsomes, this biotransformation product was not assayed in these studies. Midaizolam also effectively inhibited the CyA metabolism by liver microsomes from EE2-pretreated female rats, decreasing AM1, AM1c, AM9, and AM4N formation by 83, 88, 80, and 85%, respectively (Fig. 2B), thereby revealing that enzyme(s) from the CYP3A subfamily were involved in this increased CyA metabolism. The nature of midaizolam inhibition of CyA metabolism was determined with liver microsomes from control female rats and CyA (0.2–2 μM), in the absence or presence of the midaizolam (0–100 μM). The K_i value for AM9 formation was determined by a Dixon plot to be 27 μM (Fig. 3). Lineweaver-Burk analyses revealed that the nature of this inhibition was competitive, in accordance with literature reports.

TABLE 1

<table>
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<th>Target</th>
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<th>Antisense Primer</th>
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a Specific also to the CYP3A2 variant P-450/6f6A.
of competitive inhibition of human liver microsomal CyA metabolism by midazolam ($K_i$: 40 μM) (Pichard et al., 1990).

**Immunoinhibition of CyA Metabolism.** To further examine the involvement of P-450s 3A and 2C in CyA metabolism, polyclonal anti-rat CYP2C6 and 3A IgGs were used to inhibit the corresponding P-450s in control and EE2-treated microsomes. Preincubation of liver microsomes from control or EE2-treated rats with anti-rat CYP2C6 IgGs (10 mg IgG/nmol P-450) only slightly decreased CyA metabolism by 9 to 19% (Fig. 4A–B). Similar preincubation with anti-rat CYP3A IgGs, however, resulted in a concentration-dependent inhibition of all four CyA metabolites. When liver microsomes from untreated rats were used (Fig. 4C), 68, 62, and 66% inhibition of AM1, AM9, and AM4N, respectively, was observed at 2.5 mg IgG. At a concentration of 10 mg of antibody, the formation of AM1 and AM9 was completely inhibited, whereas the formation of AM4N was reduced by 89%. Similar inhibition of CYP3A-dependent CyA metabolism was also observed in liver microsomes from EE2-treated rats (Fig. 4D). Anti-rat CYP3A IgGs (10 mg) inhibited the formation of AM1, AM1c, AM9, and AM4N by 92, 84, 81, and 82%, respectively. In all of these studies, only 5 to 12% decreases in CyA metabolism were observed when preimmune IgGs (2.5–10 mg IgG/nmol P-450) were used as controls, with liver microsomes from untreated and EE2-treated rats.

**EE2-Mediated CYP3A9 Induction in Rat Liver.** The expression of CYP3As in control and EE2-treated female rat livers were examined using RT-PCR (Fig. 5; primer pairs, see Table 1). As seen in Fig. 6, EE2 treatment selectively induced CYP3A9 expression by 3.3-fold whereas CYP3A18 and CYP3A23 were slightly reduced by 17 and 12%, respectively, compared with untreated rats. No CYP3A2 mRNA was detected in the liver of induced or untreated female rats.

**CYP3A9 Functional Assays.** Both CYPs 3A4 and 3A9 metabolized CyA to four identifiable metabolites, albeit in different ratios (Table 3). Whereas CYP3A4 preferentially catalyzed AM1 and AM4N formation, CYP3A9 catalyzed AM9 and AM1c formation with relatively greater efficiency. On the other hand, CYP3A9 exhibited...
edited undetectable 6β-OH-testosterone formation, a functional probe for most CYPs of the 3A subfamily, unlike its CYP3A4 ortholog, which as expected exhibited pronounced testosterone 6β-hydroxylase activity (Table 3).

**Discussion**

CyA is metabolized in animal and human livers by P-450-catalyzed reactions, in which CYPs 3A are the major participants. Although over 20 different CyA metabolites have been identified so far in blood and bile, the major pathways of CyA metabolism involve monohydroxylation to the metabolites AM1, AM9, and the cyclic compound AM1c as well as N-demethylation to the AM4N metabolite (Kolars et al., 1992; Fahr, 1993). The production of each of these four CyA metabolites appears to be exclusively catalyzed by CYP3A because pretreatment of male and female rats with the CYP3A inducer DX significantly stimulated the formation of all of these metabolites, whereas preincubation of rat liver microsomes with anti-CYP3A IgGs almost completely inhibited their production (Kolars et al., 1992). On the other hand, repeated treatment of female rats with the CYP3A inactivator EE2 enhanced CyA metabolism but reduced testosterone metabolism.

Metabolite formation was measured by HPLC (methods) and expressed as the percentage of corresponding control values obtained by preimmune IgGs. 100% values correspond to 0.67, 2.57, and 1.02 pmol/mg microsomal protein/min of AM1, AM9, and AM4N for untreated, and to 0.79, 0.83, 8.49, and 1.19 pmol/mg microsomal protein/min of AM1, AM1c, AM9, and AM4N for EE2-treated female rats, respectively.

![Figure 4](image-url)
This possibility led to our present investigation of the specific role of CYPs of the 2C or 3A subfamilies in the increased CyA metabolism observed in EE2-treated female rats. Competitive inhibition experiments with the CYP3A substrate, midazolam, revealed a greater than 80% inhibition of liver microsomal formation of AM1, AM1c, AM9, and AM4N. Additionally, polyclonal anti-rat CYP3A IgGs, but not anti-rat CYP2C6 IgGs, strongly and selectively inhibited the formation of all four CyA metabolites, indicating that hepatic CYP3A enzymes distinct from Cyps 3A1/3A2 were responsible for the increased CyA metabolism by liver microsomes from EE2-treated rats.

To identify this (these) CYP3A enzyme(s), we studied the expression of four novel CYP3A genes in livers of control, EE2-, and DX-treated female rats using quantitative RT-PCR, with β-actin as a standard. Because no CYP3A1 mRNA was detected in these female rats (Mahnke et al., 1997), only probes for CYP3A2, CYP3A9, CYP3A18, and CYP3A23 were included. EE2 treatment was found to selectively induce CYP3A9 expression by greater than 3-fold, whereas the expression of CYP3A18 and CYP3A23 was slightly inhibited. No CYP3A2-mRNA was detected in untreated and EE2-treated female rats. These data are in accordance with the reports that a 10-fold higher expression of CYP3A9 is observed in the female rat liver than in the male rat liver (Wang and Strobel, 1997) and that CYP3A2-mRNA is undetectable in untreated adult female rats (Gonzalez et al., 1986). Previous studies also revealed that DX pre-treatment marginally increases the CYP3A9 expression in adult female rats by 1.7-fold, in contrast to the 6.6- and 3-fold increased expression of CYP3A18 and CYP3A23, respectively (Mahnke et al., 1997).

The EE2-mediated increases in AM1c and AM9 formation of 2.7- and 3.2-fold, respectively, with negligible increases (23 and 37%, respectively) in that of AM1 and AM4N, suggest that CYP3A9 predominantly catalyzes AM1c and AM9 formation. Functionally reconstituted recombinant CYP3A9 predominately metabolized CyA to AM9 and AM1c, supporting the fact that EE2-inducible CYP3A9 is indeed the P-450 enzyme responsible for the EE2-mediated stimulation of CyA metabolism. Interestingly, incubation of CYP3A9 with testosterone revealed no detectable 6β-OH-testosterone formation, in contrast to the pronounced β-OH-testosterone activity of CYP3A4. If these results are indeed valid, then to our knowledge, EE2-inducible CYP3A9 is the first enzyme of the 3A subfamily that spurns the functional hallmark of its other 3A subfamily members, by exhibiting no detectable testosterone 6β-hydroxylase activity. The possibility exists of course that the functional reconstitution conditions for CYP3A9 differ from those for CYP3A4 and other CYPs 3A, or that the N-terminal CYP3A9 His6-Tag (which we were unable to cleave off) interferes in its functional reconstitution with P-450 reductase and/or cytochrome b5. However, our finding of detectable CYP3A9-dependent CyA metabolism with the same CYP3A9 preparation at a level higher than that catalyzed by CYP3A4 (Table 3), dispels the latter concern.

In summary, our studies reveal the relevance of CYP3A9, a new member of the cytochrome CYP3A subfamily in CyA metabolism, that is EE2-inducible and possibly functionally distinct.

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References


White IH and Muller-Eberhard U (1977) Decreased liver cytochrome P-450 in rats caused by norethindrone or ethynyloestradiol. Biochem J 166:57–64.