Experimental approaches to evaluate activities of cytochromes P450 3A

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ABSTRACT
Cytochrome P450 (CYP) is a heme protein oxidizing various xenobiotics, as well as endogenous substrates. Understanding which CYP enzymes are involved in metabolic activation and/or detoxication of different compounds is important in the assessment of an individual’s susceptibility to the toxic action of these substances. Therefore, investigation which of several in vitro experimental models are appropriate to mimic metabolism of xenobiotics in organisms is the major challenge for research of many laboratories. The aim of this study was to evaluate the efficiency of different in vitro systems containing individual enzymes of the mixed-function monoxygenase system to oxidize two model substrates of CYP3A enzymes, exogenous and endogenous compounds, α-naphthoflavone (α-NF) and testosterone, respectively. Several different enzymatic systems containing CYP3A enzymes were utilized in the study: (i) human hepatic microsomes rich in CYP3A4, (ii) hepatic microsomes of rabbits treated with a CYP3A6 inducer, rifampicine, (iii) microsomes of Baculovirus transfected insect cells containing recombinant human CYP3A4 and NADPH:CYP reductase with or without cytochrome b5 (Supersomes™), (iv) membranes isolated from of Escherichia coli, containing recombinant human CYP3A4 and cytochrome b5, and (v) purified human CYP3A4 or rabbit CYP3A6 reconstituted with NADPH:CYP reductase with or without cytochrome b5 in liposomes. The most efficient systems oxidizing both compounds were Supersomes™ containing human CYP3A4 and cytochrome b5. The results presented in this study demonstrate the suitability of the supersomal CYP3A4 systems for studies investigating oxidation of testosterone and α-NF in vitro.

KEY WORDS: cytochrome P450 3A; α-naphthoflavone; metabolism

INTRODUCTION
The cytochrome P450s (CYP) are a family of hemoprotein enzymes that play important roles in the metabolism of drugs and carcinogens, as well as endogenous compounds such as prostaglandins, fatty acids and steroids (Gonzalez and Gelboin, 1992, Ortiz de Montellano 1995).

CYP3A is one of the major subfamilies expressed in human livers and is found at high levels in the intestinal tract (Hosea et al., 2000). This enzyme oxidizes endogenous and exogenous compounds as well as over half of the drugs in therapeutic use (Hosea et al., 2000). The human CYP3A subfamily expressed in human livers consists of CYP3A4 (Beaune et al., 1986), CYP3A5 (Aoyama et al., 1989, Yamaori et al., 2003), and CYP3A7 (Kitada et al., 1985, Yamaori et al., 2003). CYP3A4 is the most abundant form of CYP3A (~30% of total CYP) expressed in adult human livers (Shimada et al., 1994). CYP3A enzymes are induced by rifampicin (RIF) in the human (CYP3A4/5) and rabbit (CYP3A6), but not in rat (CYP3A1/2). CYP3A4 demonstrates homotropic cooperativity (non-Michaelis-Menten kinetics) with a number of substrates (Atkins 2005, Ekins et al., 2003, Guengerich 1999, Tsalkova et al., 2007, Ueng et al., 1997). The enzyme is also known to exhibit heterotropic cooperativity, which is characterized by increased oxidation of one substrate in the presence of an effector, such as α-naphthoflavone (α-NF), that may also serve as a substrate or inhibitor (Galetin et al., 2002, Harlow and Halpert 1998, Koley et al., 1997, Shou et al., 1994, Tang and Stearns 2001, Tsalkova et al., 2007). During the past decade understanding of the mechanism of CYP3A4 cooperativity has progressed from a static model with multiple binding sites (Donemski et al., 1998, Shou et al., 1994, Tsalkova et al., 2007, Ueng et al., 1997) to more complex dynamic model suggesting effector-induced conformational rearrangements of the enzyme along with multiple ligand binding (Atkins et al., 2001, Ison and Guengerich 2006).

CYP3A4 cooperativity may be influenced by the levels of the redox partners, such as cytochrome b5 or cumene hydroperoxide, relative to the CYP (Kumar et al., 2005, Ueng et al., 1997). Cytochrome b5 has been reported to stimulate CYP3A4, dependent on the specific substrate (Kumar et al., 2005, Patki et al., 2003, Yamazaki et al., 1999).
The mode of action of cytochrome b5 remains controversial (Kumar et al., 2005). Although the role of this protein as a source of electrons for CYPs is well known (Guryev et al., 2001, Schenkmann and Jansson 1999, Yamaaki et al., 2001), increasing evidence points to an allosteric effects of cytochrome b5 mediated in part by an effect on the CYP spin state (Reed and Hollenberg 2003a, Reed and Hollenberg 2003b). Its modulatory effect is further supported by the fact that cytochrome b5 not only increases CYP activity but in some cases also inhibits its activity (Reed and Hollenberg 2003b, Yamaori et al., 2003). In addition, the interaction with cytochrome b5 may affect the degree of oligomerization of CYP in membrane (Yamada et al., 1995).

Understanding which CYP enzymes are involved in the metabolic activation and/or detoxification of xenobiotics and endogenous compounds is important in the assessment of an individual’s susceptibility to the toxic action of these substances. Therefore, investigation which of several in vitro experimental models are appropriate to mimic metabolism of xenobiotics in organisms is the major challenge for research of many laboratories.

The aim of the present work was to evaluate the efficiency of different in vitro systems containing individual enzymes of the mixed-function monoxygenase system to oxidize two model substrates of CYP3A, exogenous (α-NF) and endogenous (testosterone) compounds, respectively.

**MATERIALS AND METHODS**

**Chemicals**

Glucose-6-phosphate, NADP+, NADPH, α-NF, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate (CHAPS), dilauroyl phosphatidylcholine, and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Testosterone and 6β-hydroxytestosterone were purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate dehydrogenase was from Serva (Heidelberg, Germany). Bicinchoninic acid was from Pierce (Rockford, IL, USA). All chemicals were of a reagent grade or better.

**Animals and pretreatment**

Adult male rabbits (2.5–3.0 kg, VELAZ, The Czech Republic) were fed ad libitum on pellet chow and water one week before treatment. Then, rabbits were pretreated with RIF (50 mg/kg in 40 mM NaOH i.p. for 3 consecutive days) and used for isolation of microsomes.

**Preparation of microsomes, isolation of enzymes and assays**

Microsomes were isolated from livers of rabbits pretreated with RIF as described previously (Stiborova et al., 1995, Stiborova et al., 1990) and stored in 0.5 ml aliquots in liquid nitrogen until use. CYP3A6 was isolated from liver microsomes of rabbit induced by RIF. The procedure was analogous as described previously (Haugen and Coon 1976, Yang et al., 1985). Rabbit liver NADPH:CYP reductase was purified as described earlier (Yasukochi et al., 1979). Protein concentrations were measured using the bicinchoninic acid protein assay with serum albumin as a standard (Wiechelman et al., 1988). Total CYP content was measured based on complex of reduced CYP with CO (Omur and Sato 1964). Supersomes™ were from Gentest corp. (Woburn, MA). Membranes isolated from of Escherichia coli, containing human CYP3A4 was a gift from Dr. Soucek (National Institute of Public Health, Prague, Czech Republic). Purified human CYP3A4 was from Prof. Anzenbacher (Palacky University, Olomouc, Czech Republic) and human microsomes from Dr. Szotakova (Charles University, Hradec Kralove, Czech Republic).

**Testosterone 6β-hydroxylation**

The incubation mixtures for measuring the testosterone metabolism contained in a final volume of 0.5 ml: 0.1 M potassium phosphate buffer, pH 7.4, 50 µM testosterone (2 µl of stock methanol solution per incubation), 10 mM MgCl2, 10 mM D-glucose 6-phosphate, 1 mM NADP+, 1 U/ml D-glucose 6-phosphate dehydrogenase and one from the used enzyme system: (i) human hepatic microsomes (0.2 µM CYP), (ii) hepatic microsomes of rabbits treated with a CYP3A6 inducer, rifampicine (0.2 µM CYP), (iii) microsomes of Baculovirus transfected insect cells containing recombinant human CYP3A4 (0.05 µM) and NADPH:CYP reductase with or without cytochrome b5 (0.2 µM) (Supersomes™), (iv) microsomes isolated from of Escherichia coli, containing recombinant human CYP3A4 (0.05 µM) and cytochrome b5 (0.2 µM) reconstituted with NADPH:CYP reductase (0.05 µM), and (v) purified human CYP3A4 (0.05 µM) or rabbit CYP3A6 (0.2 µM) reconstituted with NADPH:CYP reductase (0.05 or 0.2 µM) with or without cytochrome b5 (0.2 or 0.8 µM) in liposomes. Microsomes and Supersomes™ were diluted on the concentration mentioned above. Bacterial membranes were reconstituted 10 min with NADPH:CYP reductase and cytochrome b5 and then diluted with buffer on the used CYP concentration (see above). Reconstitution of purified CYP3A4 and CYP3A6 with NADPH:CYP reductase was carried out essentially as described earlier (Burke et al., 1985). Briefly, CYP3A were reconstituted as follows (0.5 µM CYP3A, 0.5 µM NADPH:CYP reductase, 0.5 µg/µl CHAPS, 0.1 µg/µl vesicles (from D,L-dilauroylphosphatidylincholine), 3 mM reduced glutathione and 50 mM HEPES/KOH, pH 7.4). An aliquot containing 25 pmol of reconstituted CYP3A4 or 100 pmol of reconstituted CYP3A6 was added to incubation mixtures. The mixtures were incubated for 15 min, at 37 °C in a shaking incubator. The reaction was terminated by addition of 0.1 ml of 1 M aqueous Na2CO3 containing 2 M NaCl. Then, phenacetin (5 µl of 1 mM stock solution) was added as an internal standard. The metabolites were extracted with 2 ml of CH3Cl2 and the extracts were evaporated to dryness. The residues were dissolved in the mobile phase for HPLC (see below).

**HPLC conditions**

Testosterone and its metabolites were separated on Nucleosil (C18) HPLC column (4.6 x 25 mm, 5 µm, Macherey-Nagel, Germany). The flow rates, mobile phases and detection wavelengths for assays were 0.6 ml/min, 70:30 CH3OH/H2O (v/v), and 254 nm, respectively.
Oxidation of α-NF

Incubation mixtures contained in a final volume of 0.5 ml: 0.1 M potassium phosphate buffer, pH 7.4, 150 µM α-NF (2 µl of stock methanol solution per incubation), 50 µl of NADPH-generating system (see above) and the same enzyme systems as in the case of testosterone oxidation (see above). The mixtures were incubated for 30 min at 37 °C in a shaking incubator. The reaction was terminated by addition of 0.1 ml of 1 M aqueous Na2CO3 containing 2 M NaCl. The α-NF metabolites were extracted with 2 ml of CH2Cl2 and the extracts were evaporated to dryness. The residues were dissolved in the mobile phase for HPLC. Samples were analyzed by HPLC as described elsewhere (Hosea et al., 2000, Thakker et al., 1981) to identify α-NF oxidation products. Two metabolites with retention times of 13.0 and 21.0 min (Figure 2), which were previously assigned as the trans-7,8-dihydrodiol and 5,6-epoxide were formed (Andries et al., 1990, Hosea et al., 2000, Shou et al., 1994). Mass spectroscopy (MALDI-TOF using α-cyano-4-hydroxycinnamic acid as a matrix) of the metabolite with a retention time of 13.0 min gave molecular ions at m/z 307 (M+H)+ and m/z 329 (M+Na)+, suggesting a dihydrodiol derivative. The metabolite with a retention time of 21.0 min gave molecular ions at m/z 289 (M+H)+ and m/z 311 (M+Na)+ and peaks at m/z 273 (M+H)+ and at m/z 295 (M+Na)+, that is an indication of an epoxide metabolite. The results are consistent with a previous studies on the metabolism of α-NF by rat microsomes pretreated with 3-methylcholanthere (Andries et al., 1990) and by purified reconstituted CYP3A4 (Shou et al. 1994), in which these two metabolites were identified as trans-7,8-dihydrodiol (retention time = 13.0 min) and 5,6-epoxide (retention time = 21.0 min). Another minor metabolite eluted with retention time of 9.6 min has not been identified yet (Figure 2). Two peaks eluted with retention times of 26.6 and 28.2 min seem not to be the products of α-NF oxidation, because they are also present in the control reaction samples. These samples containing all reaction components were immediately (without incubation) applied on a HPLC column to analyze.

RESULTS

Several different systems containing CYP3A enzymes were utilized to investigate oxidation of testosterone and α-NF in vitro: (i) human hepatic microsomes rich in CYP3A4, (ii) hepatic microsomes of rabbit treated with CYP3A6 inducer, rifampicine, (iii) microsomes of Baculovirus transfected insect cells containing recombinant human CYP3A4 and NADPH:CYP reductase with or without cytochrome b5 (Supersomes™), (iv) membranes isolated from of Escherichia coli, containing human CYP3A4 with or without cytochrome b5 and (v) purified human CYP3A4 or rabbit CYP3A6 reconstituted with NADPH:CYP reductase with or without cytochrome b5 in liposomes.

The most efficient systems oxidizing testosterone to its 6β-hydroxylated metabolite were Supersomes™ containing human CYP3A4 and cytochrome b5 (Figure 1), while low efficiency of CYP3A4 and/or CYP3A6 expressed in membranes of E. coli or reconstituted with NADPH:CYP reductase in liposomes were found (Figure 1). The activity of purified CYP3A4 or CYP3A6 reconstituted with NADPH:CYP reductase (measured as testosterone 6β-hydroxylation) was enhanced by cytochrome b5. This protein did not influence oxidation of testosterone by recombinant CYP3A4 of E. coli membranes.

α-NF was oxidized into two metabolites, tentatively identified as trans-7,8-dihydrodiol (retention time = 13.0 min) and 5,6-epoxide (retention time = 21.0 min) by mass spectra and previously reported data (Andries et al., 1990, Shou et al., 1994) (Figure 2). Under the condition used, 5,6-epoxide was the major product in all systems used. The most efficient system oxidizing α-NF was Supersomes™ containing human CYP3A4 and cytochrome b5 (Figure 3). Also low efficiency...
of CYP3A4 and/or CYP3A6 expressed in membranes of E. coli or reconstituted with NADPH:CYP reductase in liposomes to oxidize α-NF were found (Figure 3). Their activity was enhanced by cytochrome b₅, while cytochrome b₅ did not influence α-NF oxidation by recombinant CYP3A4 of E. coli membranes.

Surprisingly, although 6β-hydroxylation of testosterone catalyzed by recombinant human CYP3A4 and NADPH:CYP reductase without cytochrome b₅ in Supersomes™ was very effective, α-NF oxidation was low in this system (Figures 1 and 3).

DISCUSSION

In this report we evaluated CYP3A activity in different enzymatic systems (microsomal and/or reconstituted systems). Under the condition used, the most efficient systems oxidizing testosterone to its 6β-hydroxylated metabolite and α-NF to trans 7,8-dihydrodiol- and 5,6-epoxide were Supersomes™ containing human CYP3A4 and cytochrome b₅. It has been shown that some CYP3A4 activities are dependent on cytochrome b₅, specific lipid mixtures, cholate, buffer and salt composition (Shimada and Yamazaki 1998, Yamazaki et al., 1996). The results of this study clearly show that the effect of cytochrome b₅ on CYP3A4-mediated oxidation of testosterone was different from that on oxidation of α-NF. This protein seems to be essential for the effective conversion of α-NF to its metabolites. Namely, 6β-hydroxylation of testosterone catalyzed by recombinant human CYP3A4 and NADPH:CYP reductase in Supersomes™ was effective even without cytochrome b₅. On the contrary α-NF oxidation by this enzymatic system was negligible without cytochrome b₅.

The results presented in this study demonstrate the suitability of the supersonal CYP3A4 systems for studies investigating oxidation of testosterone and α-NF in vitro.

The data indicate that results found in a variety of studies employing different enzymatic systems to evaluate the degree of participation of individual CYP enzymes in xenobiotic metabolism should be carefully interpreted. In order to properly evaluate a role of individual CYPs in metabolism, combination of various in vitro and in vivo approaches should be utilized.

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