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Generation of specific antibodies and their use to characterize sex differences in four rat P450 3A enzymes following vehicle and pregnenolone 16α-carbonitrile treatment

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Abstract

The purpose of this study was to identify isozyme-specific antibodies and use them to determine the expression levels of four P450 3A enzymes in the livers of vehicle- and pregnenolone 16α-carbonitrile (PCN)-treated rats of both sexes, since previous work on mRNA levels has shown considerable sexual dimorphism. Using Western blot analysis with four isozyme-specific antibodies, we show that P450 3A1, 3A2, and 3A9 were expressed in vehicle-treated adult female rats at very low levels whereas P450 3A18 was not detected. PCN treatment of females strongly induced the expression of P450 3A1 in the livers with protein product increases of 214-, 3-, and 5-fold for P450 3A1, 3A2, and 3A9, respectively, and P450 3A18 was induced to 3.7 pmol/mg protein. In contrast, all four P450 3As were detected in livers of vehicle-treated males, in the order of 3A2 ≫ 3A18 > 3A9 ≅ 3A1. The protein product increases induced by PCN treatment of male rats were 92-, 3-, 6-, and 16-fold for P450 3A1, 3A2, 3A9, and 3A18, respectively.

Keywords: CYP3A; Cytochrome P450 3A; P450 3A1; P450 3A2; P450 3A9; P450 3A18; Specific antibody; Quantitative Western blot; Baculovirus expression system; Testosterone metabolism; Pregnenolone 16α-carbonitrile

Cytochrome P450 (CYP)[1] is a superfamily of hemoproteins responsible for the biotransformation of many exogenous and endogenous chemicals. The P450 3A subfamily metabolizes a wide array of therapeutic agents, carcinogens, as well as endogenous compounds [1]. It has been suggested that more than 50% of the therapeutic agents on the market are metabolized, albeit to varying extents, by P450 3A enzymes [2]. Rodent models have been extensively used in pharmacological and toxicological studies, and P450 3A enzymes play an important role in the metabolism of drugs and xenobiotics. P450 3A enzymes are found in large amounts in rodent liver but are also detected at much lower levels in extrahepatic tissues including brain, intestine, kidney, lung, leukocytes etc. [3–8]. The expression level of P450 3A enzymes is often highly variable. This variability is due to multiple factors, including gender, genetic factors, and induction or repression by certain therapeutic agents and some xenobiotics.

To date, at least six cDNAs (CYP3A1, CYP3A2, CYP3A9, CYP3A18, CYP3A23, and CYP3A62) of the CYP3A subfamily have been described in the liver of rats [5,9–13]. CYP3A1[2] and CYP3A23 are highly homologous

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[1] *Abbreviations used:* P450 or CYP, cytochrome P450; PCN, pregnenolone 16α-carbonitrile; RT-PCR, reverse transcription polymerase chain reaction; PAb, polyclonal antibody; MAb, monoclonal antibody; NPOR, NADPH-cytochrome P450 oxidoreductase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MOI, multiplicity of infection; HPLC, high performance liquid chromatography.

[2] We use "CYP3A1" to refer to CYP3A1 and/or CYP3A23, based on the original product name from Gentest Corp, and use P450 3A1 to refer to the protein.

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(98% identity in DNA sequences) and have been suggested to be allelic variants [14,15]. The expression of rat CYP3As is gender-, tissue-, and developmental-dependent and is regulated by several chemicals including pregnenolone 16α-carbonitrile (PCN), dexamethasone, clotrimazole, and phenobarbital [8,12,14,16,17]. In the liver of adult rats, P450 3A1 protein is absent or expressed at very low levels but is highly inducible by glucocorticoids such as dexamethasone [17,18]. P450 3A2 protein is expressed constitutively in adult male rats, only modestly induced by glucocorticoids, and possibly more effectively by phenytoin [19,20]. Studies on mRNA levels revealed that CYP3A9 is a female-predominant form [14,16] whereas CYP3A18 is a male-specific form in adult rat liver [12,14]. Recently, CYP3A62 was discovered in the rat liver and found to be a major P450 3A form in the small intestines of both sexes [13].

Using specific primers for reverse transcription-polymerase chain reaction (RT-PCR), the expression levels of individual CYP3A mRNAs have been investigated in control and xenobiotic-treated rats [14,21]. The protein level of each isoform, however, is not well characterized due to lack of specific probes. Traditionally, P450 3A enzymes in tissues may be identified and measured in vitro in two ways: measuring enzyme activity using probe substrates, or measuring P450 apoprotein levels using specific monoclonal or polyclonal antibodies. The catalytic markers such as the activity of testosterone 6β-hydroxylase [22,23], midazolam 4-hydroxylase [24,25], and quinine 3-hydroxylase [26] have been used to monitor the activity of hepatic P450 3A enzymes. However, the proportion of enzyme activity attributable to individual P450 3A enzymes is currently unknown due to a lack of specific probe substrates for each enzyme of the P450 3A subfamily. On the other hand, monoclonal antibodies and/or polyclonal anti-peptide antibodies which target unique sequences can recognize a single member of a highly related family of proteins and thus provide the means to measure an individual member of the P450 3A subfamily as has been shown with other related P450s [17,27–30].

PCN is a synthetic steroid derivative and an anti-glucocorticoid. PCN is characterized as a rat P450 3A prototypical inducer. Early research by Lu et al. [31] on PCN revealed that PCN induced drug-metabolizing activities in rat livers. Hans Selye's laboratory characterized PCN as a catatoxic steroid [32] since pretreatment of rats with PCN protected them from adverse effect of certain chemicals. This protection effect of PCN was due in part by inducing drug-metabolizing activities in the liver and possibly in extrahepatic tissues as well. Further studies demonstrated that PCN induced CYP3A subfamily enzymes, predominately P450 3A1 [4,9,17]. The induction occurred at both the levels of mRNA as well as protein. However, many studies were conducted before the availability of isoform-specific antibodies for individual P450 3A enzymes. These early studies could not determine the induction or the levels of individual P450 3A isoforms following PCN treatment.

In a previous work, we isolated several isozyme-specific monoclonal antibodies made against purified P450p (P450 3A1) and P450l (P450 3A2) [17]. However, the reactivity with P450 3A9 and P450 3A18 was unclear at that time because there were no standards. In the present study, we utilized the baculovirus expression system to produce recombinant P450 3A9 and P450 3A18 to be used as standards. We also produced polyclonal anti-peptide antibodies by immunizing rabbits with peptides representing certain regions of P450 3A9 and 3A18. Using heterologously expressed individual P450s from rat P450 3A subfamily as well as 12 additional expressed P450s, we identified antibodies which are specific for each of the four rat CYP3A isozymes. Then, we used these specific antibodies to examine to what extent PCN treatment, which is known to induce P450 3A related catalytic activity and mRNA to a similar extent, would alter the hepatic expression of the four rat P450 3As. The expression levels of four P450 3A enzymes were measured in liver microsomal preparations in adult rats of both genders with and without PCN treatment, and P450 3A enzymatic activity was determined with testosterone as substrate.

Materials and methods

Materials

The female rat cDNA library, ExAssist helper phage, SOLR cells, and picoBlue Immunoscreening kit were purchased from Stratagene (La Jolla, CA). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Restriction enzymes were purchased from New England BioLab (Beverly, MA). The GeneAmp RNA PCR core kit and *Taq* polymerase were purchased from Perkin-Elmer (Norwalk, CT). The Bac-to-Bac baculovirus expression system, DH10Bac cells, TRIzol reagent, Grace's insect medium, fetal bovine albumin, antibiotics, and agarose were purchased from Gibco (Gaithersburg, MD). Sf21 cells and TA Cloning kit were purchased from Invitrogen (Carlsbad, CA). The QIAquick gel extraction kit and QIAGEN plasmid mini kit were obtained from Qiagen (Valencia, CA). CYPs 1A1, 1A2, 2A1, 2A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2D1, 2D2, 2E1, 3A1, and 3A2 Supersomes were purchased from Gentest (Woburn, MA). Cytochrome b_5 was purchased from Panvera (Madison, WI). Goat anti-mouse IgG- and goat anti-rabbit IgG-conjugated alkaline phosphatase were obtained from Sigma Chemical (St. Louis, MO). PCN was obtained from Biomol Research Lab (Plymouth Meeting, PA). Testosterone was purchased from Fisher Scientific (Pittsburgh, PA). Mono-hydroxylated metabolites of testosterone and 11 α-hydroxyprogesterone were purchased from Steraloids (Wilton, NH). All the other chemicals were molecular biology grade, certified A.C.S., or reagent grade analyzed chemicals from commercial sources.