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SHORT COMMUNICATION

Developmental Changes in the Constitutive and Inducible Expression of Cytochrome P450 3A2

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ABSTRACT. Using a CYP3A2-specific oligonucleotide and an antipeptide antibody raised against the C terminus of CYP3A2 (VINGA) it is demonstrated that metyrapone administration to adult (12 weeks old) but not immature (3 weeks old) male Sprague Dawley rats induces the hepatic expression of CYP3A2 mRNA and protein. The constitutively expressed level of CYP3A2 protein in adult male rats is markedly lower than the levels expressed in immature rats as determined using the anti-VINGA antibody, in contrast to previous reports using antibodies that do not discriminate between CYP3A forms. Hepatic microsomal CYP3A2 protein expression, examined between 3 and 15 weeks of age, is extinguished between 9 and 12 weeks of age in contrast to immunoreactive CYP3A protein (determined using a nonselective antibody) and CYP3A-dependent androstenedione 6β-hydroxylase activity. These data suggest that the regulation of the induction of CYP3A2 is developmentally controlled and that the major expressed adult form(s) of constitutively expressed CYP3A is not CYP3A2. BIOCHEM PHARMACOL 54;7:841–846, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cytochrome P450; steroid 6β-hydroxylase; CYP3A2; rat liver; gene expression; metyrapone

The cytochrome P450 (CYP)‖ 3A subfamily is a constitutively highly expressed CYP subfamily in both rats and humans and is prominent in the metabolism of many drugs and endogenous compounds [1]. The levels of expression are modulated by glucocorticoids, organochlorine pesticides, and several drugs [2, 3], although the mechanisms involved are not well defined.

More than four CYP3A genes may be expressed in the rat liver. The closely related CYP3A1 and CYP3A23 are constitutively lowly expressed genes transcriptionally induced by glucocorticoids and other compounds such as phenobarbitone [4–7]; CYP3A2 is reported to be the prominently expressed form in adult male rats [8–10]; CYP3A9 cDNA has recently been cloned from rat brain [11] and is also expressed in liver¶; and CYP3A18 cDNA has been cloned from female liver but is more prominently expressed in male liver [12].

Determining the effect of modulators on the expression of CYP3A proteins is complicated by difficulties in separating closely related proteins by conventional electrophoretic methods [9, 10]. Therefore, form-specific oligonucleotides have been used to determine the levels of expression of each mRNA in response to inducers. These studies indicated that glucocorticoids markedly induce CYP3A1 mRNA but not CYP3A2 mRNA in the livers of treated animals [4, 7, 8]. However, recently developed monoclonal antibodies indicate that hepatic CYP3A2 protein is induced by glucocorticoid treatment (in 9-week-old rats), suggesting that post-transcriptional mechanisms of regulation exist for the CYP3A2 gene [9] and that determination of mRNA levels may not correlate with the levels of protein/enzyme activity.

The effect of metyrapone on CYP3A2 expression is examined since this compound is a transcriptional inducer of the CYP3A1/CYP3A23 genes but does not bind to or activate the glucocorticoid receptor [13]. The precise role of the glucocorticoid receptor in CYP3A regulation is as yet to be fully determined, and the use of steroidal inducers to determine the mechanism of CYP3A induction is complicated by their interaction with the glucocorticoid receptor. The effect of metyrapone on the developmental regulated inducible expression of the CYP3A2 gene was examined in both prepubertal (3 weeks old) and adult (12 weeks old)

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‖ *Abbreviations:* CYP, cytochrome P450; metyrapone, 2 methyl-1,2-bis-(3-pyridyl)-1-propanone. The nomenclature used for cytochrome P450s is that recommended by Nelson *et al.* [1]. CYP3A2 refers to the gene products of pcn2 mRNA [7] and P450/6βA [15] mRNA, which differ by 12 nucleotides leading to 2 amino acid differences in their corresponding proteins. CYP3A refers to one or more cytochrome P450 3A subfamily genes, which include CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A23.

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TABLE 1. Examination of the specificity of CYP3A2 probes within the rat CYP3A subfamily

CYP	C terminus amino acid sequence*	CYP3A2 oligo hybridisation to mRNA†	No. of mismatches for CYP3A2 oligo
CYP3A1	VPEIITGS	3'-TGGCTGAACCTTGGGTATCT-5' 5'-(+68)ACGGATTTGGGACCCGCACA(+88)-3'	6
CYP3A2‡	LPAVINGA	3'-TGGCTGAACCTTGGGTATCT-5' 5'-(+68)ACCGACTTGGAACCCATAGA(+88)-3'	0
CYP3A9	DETTVNGA	3'-TGGCTGAACCTTGGGTATCT-5' 5'-(+68)ACCTATATGGAACTCATTCA(+88)-3'	6
CYP3A18	ILKLVSRD	3'-TGGCTGAACCTTGGGTATCT-5' 5'-(+68)ACATATATGGGACCTATTCT(+88)-3'	9
CYP3A23	VPEIITGS	3'-TGGCTGAACCTTGGGTATCT-5' 5'-(+68)ACGGATTTGGGACCCGCACA(+88)-3'	6

Amino acid and nucleotide sequences were aligned using PC/GENE software (Oxford Molecular Ltd, Oxford, UK) using a program based on the method of Myers and Miller [17].

*Underlined sequence refers to immunogen used to raise antibody to CYP3A2. The VINGA sequence does not apeear elsewhere within the CYP proteins.

†Upper sequence is the oligonucletide sequence with lines indicating homology to lower CYP mRNA sequences (numbers refer to nucleotides from rhe translation start site). The CYP3A2 oligo did not show higher homology elsewhere within CYP3A mRNAs.

‡The pcn2 [7] and 6β-A [15] mRNAs are identical between nucleotides 68 and 88.

male rats because the constitutive expression of CYP3A2 is apparently independent and dependent of testosterone in prepubertal [14] and adult rats [5], respectively, although the reason(s) for this difference is unknown.

MATERIALS AND METHODS

Male and female Sprague Dawley rats were bred from animals originally supplied by Charles River (Margate, UK). Rats were maintained on diet No. 1 (SDS, Witham, Essex, UK) and water *ad libitum*. Rats were treated at 3 weeks of age (weaning) and 12 weeks of age i.p. with 100 mg of metyrapone/kg body weight dissolved in saline vehicle and sacrificed 24 hours later. Control animals received saline vehicle only. Metyrapone was purchased from Sigma Chemical Co. (Poole, Dorset, UK).

Total liver RNA was prepared using RNAzol (Biogenesis, Bournemouth, UK). For analysis of mRNA expression, RNA was electrophoresed on 1.5% (w/v) denaturing (formaldehyde) agarose gels and transferred to nylon membrane (Hybond N, Amersham UK). For examination of CYP3A2 mRNA expression, the radiolabeled oligonucleotide 5'-TCT ATG GGT TCC AAG TCG GT-3' complementary to nucleotides 68–88 of PCN2 [7] and P450/6βA [15] mRNAs was hybridized under the conditions previously outlined [5]. The oligonucleotide is not expected to hybridize to other CYP3A mRNAs under the conditions employed since there is a minimum of 6 mismatches with other rat CYP3A mRNAs in this region (see Table 1). Furthermore, this oligonucleotide does not hybridize with RNA from female adult liver [5], although adult female rat liver express CYP3A9 and CYP3A18 mRNAs at levels greater than and equal to the adult male rat liver, respectively [16]. The oligonucleotide was custom synthesized by Pharmacia and 5' end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using polynucleotide kinase (Promega).

Microsomal fractions were prepared from livers by centrifugation as previously outlined [18]. Western Blotting was performed after SDS-polyacrylamide gel electrophoresis under reducing conditions using a MiniP2 Bio-Rad electrophoresis apparatus. Protein was transferred onto nitrocellulose and blocked overnight with 3% (w/v) BSA, 0.3% (w/v) Tween 20. Polyclonal rabbit antirat cytochrome P450 reductase antibody was purchased from Amersham (UK). Rabbit polyclonal antirat CYP3A antibody [19], purified CYP3A2 [10], and purified CYP3A1 [20] were generously provided by David Waxman (Harvard Medical School, Boston, MA, USA), James Halpert (University of Arizona, AZ, USA), and Roland Wolf (Biomedical Research Centre, Ninewells Hospital and Medical School, Dundee, Scotland), respectively. Antipeptide antibodies were raised against the C termini of CYP3A1 (IITGS) and CYP3A2 (VINGA) as described previously [21]. The anti-VINGA antibody binds to CYP3A2 (translated from both the pcn2 [7] and P450/6βA [15] mRNAs) but not to CYP3A1 [21]. It is highly unlikely that the anti-VINGA

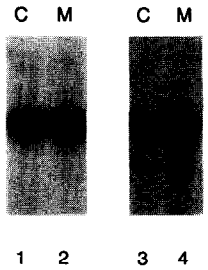

FIG. 1. Northern blot of total hepatic RNA from 3- and 12-week-old control rats and rats treated with metyrapone. 20 μg of RNA/lane was subjected to Northern analysis, and the expression of CYP3A2 mRNA levels was examined. Lanes 1 and 2, 3-week-old rats; lanes 3 and 4, 12-week-old rats. C, control; M, metyrapone-treated rats. Results are typical of three separate animals per treatment/age.

antibody will bind to CYP3A23 as the C terminus of this form is the same as CYP3A1. Also, binding to CYP3A18 is unlikely as the C terminus is different (LVSRD) from the immunizing peptide. However, the antibody may bind to CYP3A9 since the protein has a similar C terminus (TVNGA). The anti-IITGS antibody binds to CYP3A1 but not to CYP3A2 [21], and based on the structure of their C termini, it is expected to bind to CYP3A23 but not to CYP3A9 or CYP3A18 (see Table 1). After incubation with the primary antibody, the blots were incubated with horseradish peroxidase conjugated anti-rabbit IgG antibody. Detection was accomplished using chemiluminescence with the ECL kit (Amersham). Western blots were scanned using a Stratagene Eagle Eye, and data were analyzed using Phoretix software (Phoretix International, Newcastle, UK).

Androstenedione hydroxylase and ethylmorphine *N*-demethylase activities were performed as previously described [13].

RESULTS AND DISCUSSION

Induction of CYP3A2 by Metyrapone

Figure 1 indicates that the administration of metyrapone to male rats has no effect on the level of hepatic CYP3A2 mRNA in 3-week-old rats but causes an increase in hepatic CYP3A2 mRNA in 12-week-old rats. Examination of microsomal protein levels of CYP3A2 using the anti-VINGA antibody (Fig. 2) indicates that metyrapone does not increase the hepatic level of CYP3A2 in 3-week-old rats but induces the level in 12-week-old rats. In contrast, hepatic microsomal CYP3A1/CYP3A23 levels determined using the anti-IITGS antibody are markedly induced from a constitutively low level by metyrapone treatment in both ages of animal (Fig. 2). Induction of CYP3A1 by metyrapone is associated with a marked transcriptional induction of the CYP3A1 gene [13].

Constitutive Expression of CYP3A2

Figures 1 and 2 indicate that in males there are higher levels of hepatic CYP3A2 mRNA and protein, respectively,

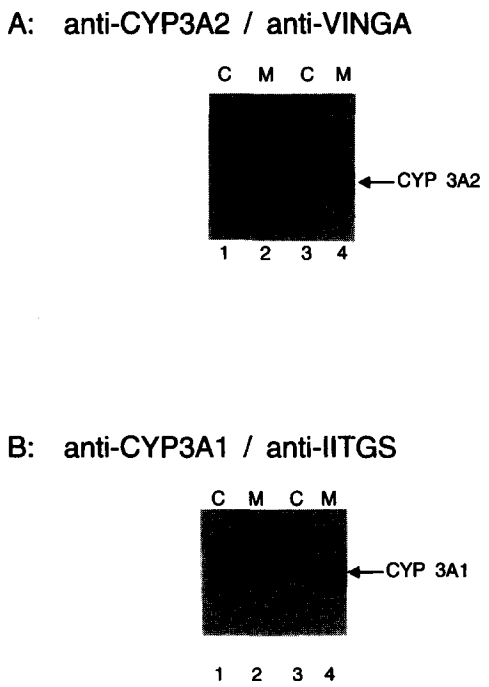

FIG. 2. Western blot of hepatic microsomal protein from 3- and 12-week-old control rats and rats treated with metyrapone. 1 μg of microsomal protein was subjected to Western analysis, and the immunoblot was probed with anti-CYP3A2/anti VINGA antibody (A) or anti-CYP3A1/anti-IITGS antibody (B). Lanes 1 and 2, 3-week-old rats: lanes 3 and 4, 12-week-old rats. C, control; M, metyrapone-treated rats. Results are typical of three separate animals per treatment/age.

in untreated 3-week-old rats compared with 12-week-old rats. This observation contrasts with previous reports that indicate a neonatal activation of CYP3A2 gene expression that is maintained in adult male rats, although expression is extinguished after 2–3 weeks of age in female rats [5, 7, 14, 19]. Examination of immunoreactive levels of microsomal CYP3A2 between the ages of 3 and 15 weeks in male and female rats indicates that immunodetectable levels of microsomal CYP3A2 protein are depressed between 3 and 6 weeks of age in females as previously indicated [14, 19] but is also depressed between 9 and 12 weeks of age in males (Fig. 3). It is possible that the antigenicity of CYP3A2 may have been masked by covalent modification, although phosphorylation is unlikely as the antigenic site contains no threonine (T), tyrosine (Y), or serine (S) residues, although sites for phosphorylation do exist elsewhere in CYP proteins [22]. Western blotting performed using an antiserum raised against CYP3A protein indicates that there is variation in the level of expression of CYP3A in both sexes throughout this period (Fig. 3). However, scanning of blots indicates that the levels of immunoreactive CYP3A do not vary by more than 20% from the level present in liver microsomes from 3-week-old rats of either sex. Figure 4 indicates that CYP3A-dependent androstenedione 6β-hydroxylase activity correlates with the levels of immunodetectable CYP3A2 in females but not in males. In contrast, in the same microsomal samples the

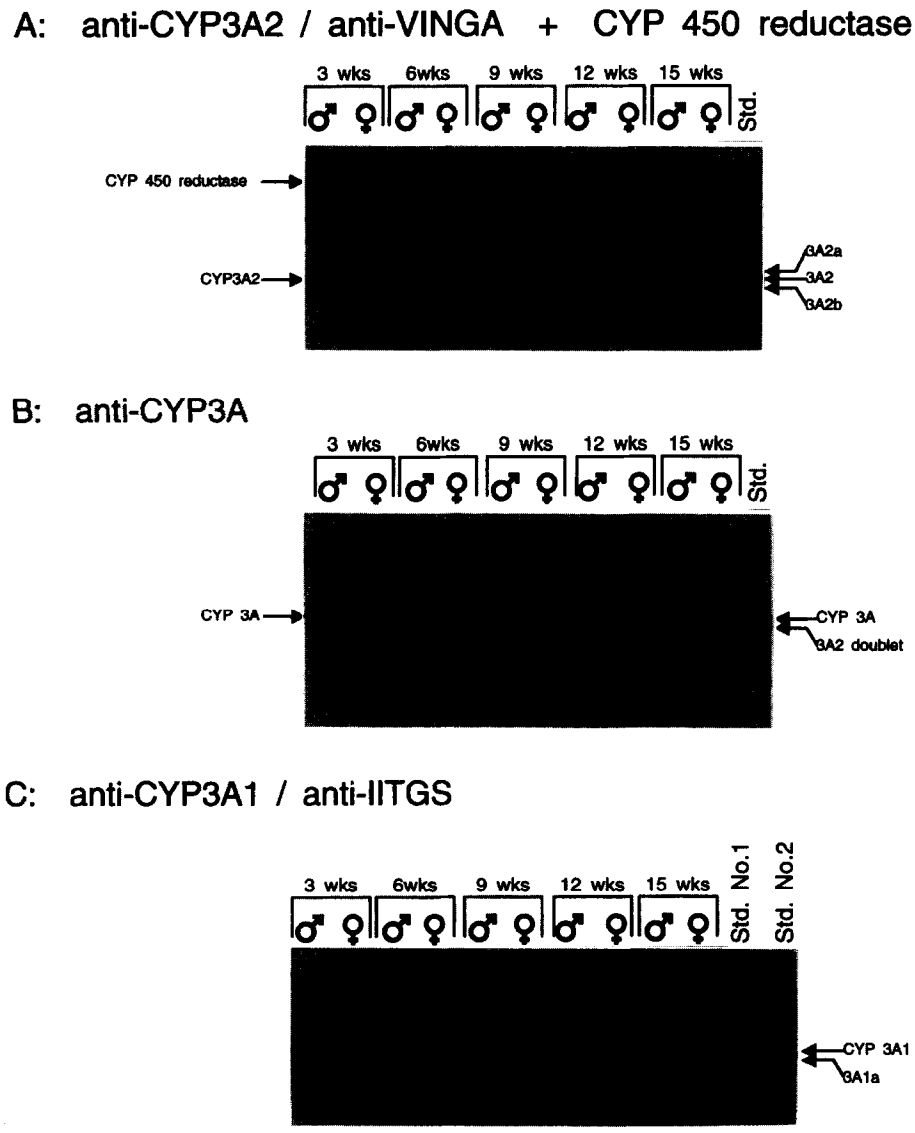

FIG. 3. Western blots of hepatic microsomal protein from 3 to 15-week-old male and female rats. 1 μg of microsomal protein was subjected to Western analysis, and the immunoblot was probed with anti-CYP3A2/anti-VINGA and anti-CYP450reductase antibodies (std, 0.5 pmol of purified CYP3A2) (A), anti-CYP3A antibody (std, 0.5 pmol of purified CYP3A2) (B), or anti-CYP3A1/anti-IITGS antibody (std 1, 0.5 pmol of purified CYP3A2; std 2, 1 pmol of purified CYP3A1) (C).

levels of other sex-dependent CYP activities (16α- and 7α-androstenedione hydroxylase activities representing CYP2C11 and CYP2A1, respectively) show a sex dependence and correlate with their respective levels of immunodetectable protein (data not shown) in agreement with previous studies [14, 19]. The lack of correlation between the developmental expression of CYP3A2 protein and androstenedione 6β-hydroxylase activity in males suggests that other CYP3A proteins also mediate this enzyme activity and constitute the major expressed CYP3A in male rats older than 9–12 weeks of age.

Constitutive Expression of Other CYP3A Proteins

Figure 3 suggests that the expression of other CYP3A genes [11, 12] is also sexually and developmentally regulated as judged by the appearance and disappearance with age of anti-VINGA, anti-CYP3A, and anti-IITGS immunoreactive proteins of similar electrophoretic mobilities to CYP3A proteins. The anti-CYP3A antibody detects a protein doublet ($3A_{doublet}$) with greater electrophoretic mobility than CYP3A1 and CYP3A2 in males at 9 and 12 weeks of age but not in females. One of these proteins may be CYP3A18/$P450_{6\beta\text{-}2}$ because the CYP3A18 mRNA seems to be prominently expressed in males but not females [12] and the $P450_{6\beta\text{-}2}$ protein is male-specific and has a higher electrophoretic mobility than CYP3A1 and CYP3A2 proteins [23]. The anti-VINGA antibody detects an additional band ($3A2_b$) with greater electrophoretic mobility than CYP3A1 and CYP3A2 in males also at 9 and 12 weeks of age and a protein ($3A2_a$) with lower electrophoretic mobility in females at 12 and 15 weeks of age (Fig. 3). One of these proteins may be CYP3A9 as the C terminus of this protein (TVNGA) [11] is similar to that of the immunizing peptide (VINGA).

These data indicate that the CYP3A2 gene is unresponsive to metyrapone induction in immature (3-week-old) male rats but is inducible at the level of mRNA and protein in adult (12-week-old) rats. This alteration in response may be associated with the apparent dependence of CYP3A2

FIG. 4. Developmental changes in hepatic microsomal CYP activities in 3 to 15-week-old male and female rats. A, androstenedione 6β-hydroxylase (●, male; ○, female) and ethylmorphine N-demethylase (■, male; □, female) activities. B, androstenedione 16α-hydroxylase (◆, male; ◇, female) and androstenedione 7α-hydroxylase (▲, male; △, female) activities. Data are the mean of three separate determinations from at least two separate animals with variation less than 15%. These data are typical of three separate studies.

expression on testosterone in adults [5] but not in prepubertal males [14]. The differences in the response of CYP3A2 to inducers and endogenous regulators may be due either to an age-dependent change in the regulatory factors for CYP3A2 or to the existence of several noncoordinately regulated CYP3A2 genes.

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