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Strain Differences in CYP3A-Mediated C-8 Hydroxylation (1,3,7-Trimethyluric Acid Formation) of Caffeine in Wistar and Dark Agouti Rats

RAPID METABOLISM OF CAFFEINE IN DEBRISOQUINE POOR METABOLIZER MODEL RATS

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ABSTRACT. We observed significant strain differences [Dark Agouti (DA) > Wistar] in 1,3,7-trimethyluric acid formation (C-8 hydroxylation) during caffeine metabolism, though not in N-demethylations, in adult male DA and Wistar rats. In contrast, adult female and immature male rats of both DA and Wistar strains did not show significant differences in activity levels of C-8 hydroxylation. Kinetic studies using liver microsomes revealed that adult male DA rats have a larger V_{max} for C-8 hydroxylation than do Wistar rats. Troleandomycin (TAO), known as a cytochrome P450 (CYP) 3A inhibitor, and an anti-rat CYP3A2 polyclonal antibody effectively reduced C-8 hydroxylation by rat liver microsomes in a concentration-dependent manner, suggesting that C-8 hydroxylation in rats is mediated largely by an isoform(s) of the CYP3A subfamily. Troleandomycin and the antibody did not inhibit the N-demethylations of caffeine by rat liver microsomes. Treatment of rats with CYP3A inducers caused a marked increase in C-8 hydroxylase activity. These results indicate that the rat CYP3A subfamily is capable of catalyzing C-8 hydroxylation of caffeine as is the case for human CYP3A4. The results of western blotting analysis using anti CYP3A antiserum showed that the staining intensity of the protein band in DA rat liver microsomes was higher than that in Wistar rat liver microsomes. We concluded that marked sex-dependent strain differences in C-8 hydroxylation of caffeine between Wistar and DA rats are due to the differences in the levels of expression of CYP3A in these strains of rats. BIOCHEM PHARMACOL **55**;9:1405–1411, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytochrome P450; caffeine metabolism; Dark Agouti rats; CYP3A

Caffeine is one of the most widely consumed dietary chemicals in the world and, therefore, constitutes an ideal model substrate for assessing xenobiotic metabolizing enzyme activity. Although caffeine metabolism is complex, the various biotransformation pathways are now well characterized. *In vivo*, caffeine biodisposition has received considerable attention over the last few years, primarily owing to its use in evaluating hepatic CYP¶. Caffeine is cleared by humans and other animals through liver metabolism by partial demethylation and hydroxylation [1], the detectable primary metabolites being not only theobromine, paraxanthine, and theophylline, but also 1,3,7-trimethyluric acid [2–7]. The primary metabolic pathways of caffeine are illustrated schematically in Fig. 1. Interspecies variations in caffeine metabolism by liver microsomes have also been studied [8]. While N-3 demethylation (paraxanthine formation) is the major pathway in humans, N-7 demethylation (theophylline formation) is predominant in monkeys, and C-8 hydroxylation (1,3,7-trimethyluric acid formation) is predominant in rats and mice. Although the caffeine metabolic profile has been shown to be different in humans and rats, in both species CYP1A2 has been seen to be involved in N-3 demethylation and N-1 demethylation (theobromine formation) [8–10]. Moreover, it has been established that in humans and rats N-7 demethylation involves P450s other than those of the CYP1A subfamily [8, 11–13]. It has also been reported that human liver microsomal C-8 hydroxylation is mediated largely by isoforms of the CYP3A subfamily [14]. The P450 isoform catalyzing C-8 hydroxylation in rats has not been reported.

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¶ *Abbreviations:* CYP, cytochrome P450; DA rat, Dark Agouti rat; and TAO, troleandomycin.

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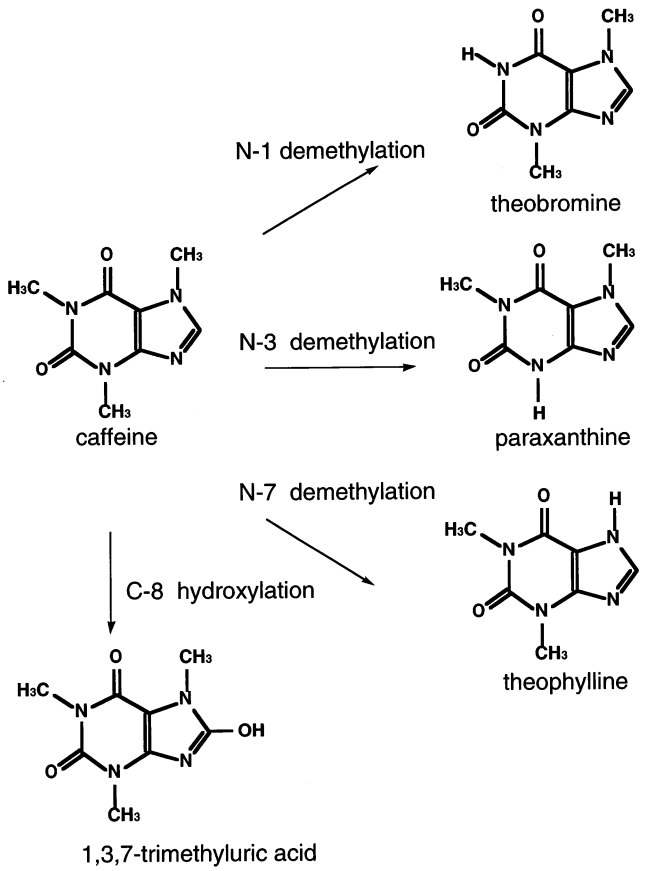

FIG. 1. Primary metabolic pathways of caffeine.

Direct comparison of caffeine metabolism between human and rat liver microsomes has been reported by Agúndez *et al.* [15]. They used DA rats in their study. The genetic deficiency in the CYP2D-related drug-metabolizing ability in this strain of rats has been studied extensively [16–18]. However, other species of cytochrome P450 have not been as well characterized in this strain of rats as in other strains of rats, such as Wistar and Sprague–Dawley rats. Therefore, caffeine metabolism by liver microsomes from the DA rat may not be typical of those from rats commonly used in drug metabolism research. In fact, we have compared caffeine metabolism in liver microsomes from DA and Wistar strains of rats and found that liver microsomes from DA rats have markedly higher C-8 hydroxylation activity than do those from Wistar rats. We investigated the age-effect on this strain difference. We used inhibitors and specific antibodies to determine the involvement of CYP3A in caffeine metabolism in rats.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: caffeine from Wako; theobromine, theophylline, and 8-chlorotheophylline from Tokyo Kasei; paraxanthine and 1,3,7-trimethyluric acid from Funakoshi; TAO, 3-methylcholanthrene, phenobarbital, dexamethasone, and pregnenolone 16α-carbonitrile from the Sigma Chemical Co.; anti-rat CYP3A2 serum for inhibition studies from the Daiichi Pure Chemical Co.; and glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH from the Oriental Yeast Co. Other chemicals and solvents were of analytical grade.

Preparation of Microsomes

Male and female Wistar rats (3 and 7 weeks old) and male and female DA rats (3 and 7 weeks old) were obtained from the Nihon SLC Co. The rats were decapitated, and their livers were excised and perfused with ice-cold 1.15% KCl (w/v). Liver microsomes were prepared according to the method of Omura and Sato [19]. The livers were homogenized in 3 vol. 1.15% KCl. After each homogenate was centrifuged at 9,000 *g* for 20 min, the supernatant was centrifuged at 105,000 *g* for 1 hr and then each pellet was homogenized with 25 mL of 1.15% KCl, followed by centrifugation at 105,000 *g* for 1 hr. The microsomes thus obtained were homogenized with 0.1 M of potassium phosphate buffer (pH 7.4), frozen by liquid nitrogen, and then stored at −80°. Protein concentrations were assayed by the method of Lowry *et al.* [20]. Total microsomal cytochrome P450 was quantified from the CO difference spectrum of the dithionite-reduced proteins between 490 and 450 nm, using an extinction coefficient of 91 mM^{-1} cm^{-1} [19].

Drug Treatments

Male Wistar rats (7 weeks old) were treated with 3-methylcholanthrene (40 mg/kg/day, i.p., 3 days), phenobarbital (80 mg/kg/day, i.p., 3 days), dexamethasone (100 mg/kg/day, i.p., 3 days), or pregnenolone 16α-carbonitrile (25 mg/kg/day, i.p., 3 days). The rats were decapitated 24 hr after the final treatment. Liver microsomes of DA rats treated with drugs were similarly obtained.

Caffeine Metabolism

The standard assay mixture contained 10 mM of caffeine, 10 mM of $MgCl_2$, 0.5 mM of NADPH, 10 mM of glucose-6-phosphate, 2 enzyme units of glucose-6-phosphate dehydrogenase, and 0.1 M of potassium phosphate buffer (pH 7.4) in a final volume of 0.5 mL. For kinetic studies, substrate concentrations of 0.5 to 32 mM were used. The reaction was started after 5 min of preincubation at 37° by the addition of 0.5 mg of microsomal protein; mixtures were incubated in a shaker bath at 37° for 10 min, and the reaction was halted by the addition of 400 μL of 10% $ZnSO_4$ followed by cooling on ice. 8-Chlorotheophylline (internal standard) was added to each sample, which was then saturated with ammonium sulfate and extracted with ethyl acetate. The extracts were dried and resuspended in 50 μL of the HPLC mobile phase.

HPLC Analysis of Caffeine Metabolites

The method is a modification of the isocratic-HPLC method described by Agúndez *et al.* [15]. The HPLC conditions were as follows: instruments, a Shimadzu LC-6A liquid chromatograph equipped with an SPD-6AV spectrophotometric detector and a data processor (Chromatopac C-R6A); the HPLC column used was Inertsil ODS (4.6 mm i.d. × 250 mm) (GL Science Inc.); the mobile phase was a mixture of tetrahydrofuran:acetic acid:acetonitrile:water (6.66:2.74:62:700, by vol.); flow rate, 1.5 mL/min; the wavelength of detection absorbance was set at 280 nm. Retention times for theobromine, paraxanthine, theophylline, 1,3,7-trimethyluric acid, caffeine, and 8-chlorotheophylline (the internal standard) were, 4.9, 6.1, 6.8, 7.3, 12, and 16 min, respectively.

Inhibition Studies

The effects of TAO on caffeine metabolism were determined. TAO was dissolved in methanol. The final concentration of methanol in the incubation mixture was 1% (v/v); an equivalent amount was added to the control incubations. TAO requires NADPH-dependent complexation for inactivation and is called a mechanism-based inhibitor. Incubation mixtures containing TAO were preincubated in the presence of the NADPH-generating system at 37° for 15 min, and the reaction of the caffeine metabolism was initiated by addition of the substrate. Immunoinhibition studies with anti-rat CYP3A2 were carried out by preincubating antiserum with microsomes (0.1 mg of protein) at room temperature for 30 min prior to the measurement of caffeine metabolite formation. Control incubations contained an equivalent amount of preimmune rabbit serum proteins.

Immunological Study

Liver microsomal proteins from adult Wistar and DA rats of both sexes were separated by electrophoresis in a 10% SDS-polyacrylamide gel [21]. The immunoblot technique was applied according to Towbin *et al.* [22], with some modifications. Anti P450 3A2 antiserum (rabbit) was used as the first antibody at a dilution of 1:500. The second antibody was goat anti-rabbit IgG (E. Y. Labs, Inc.), at a dilution of 1:100. The third antibody was rabbit peroxidase anti-peroxidase IgG (Jackson Immuno Labs, Inc.), at a dilution of 1:5000. The peroxidase activity was detected by using 10 mg of diaminobenzidine in 40 mL of 50 mM of Tris-HCl buffer (pH 7.4) and H_2O_2 at a final concentration of 0.0075%.

Data Analysis

Enzyme kinetic parameters (K_m and V_{max}) were analyzed according to the Michaelis–Menten equation, using a non-linear regression program (Simplex) [23]. Statistical significance was calculated by Scheffe's test. Differences were considered to be significant when $P < 0.05$.

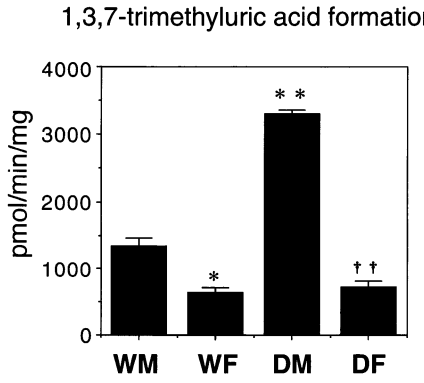

FIG. 2. Sex and strain differences in C-8 hydroxylation (1,3,7-trimethyluric acid formation) of caffeine metabolism in young adult rat liver microsomes. Activities of caffeine metabolism by liver microsomes from 7-week-old Wistar male (WM), Wistar female (WF), DA male (DM), and DA female (DF) rats were determined. Incubation was carried out at 37° for 10 min with 10 mM of caffeine. Other experimental details are described under Materials and Methods. Data represent means ± SEM of four animals. * and **Significantly different from WM ($P < 0.05$ and $P < 0.01$, respectively); ††Significantly different from DM ($P < 0.01$).

RESULTS AND DISCUSSION

Caffeine *N*-demethylase and C-8 hydroxylase activities were assayed in liver microsomes from Wistar and DA rats of both sexes (7 weeks old). We determined the amounts of theobromine (N-1 demethylation), paraxanthine (N-3 demethylation), theophylline (N-7 demethylation), and 1,3,7-trimethyluric acid (C-8 hydroxylation) by HPLC after incubating caffeine with rat liver microsomes.

The main metabolite of caffeine in rats was 1,3,7-trimethyluric acid (C-8 hydroxylation formation), which amounted to more than 80% of the total caffeine metabolites. The three demethylation reactions were minor and about equal. These results agreed with results published previously [8]. As Fig. 2 shows, significant strain differences (DA male > Wistar male) and sex differences (male > female) were observed in the C-8 hydroxylation. In contrast, the three *N*-demethylase activities were higher in male than in female rats of corresponding strains, but significant strain differences were not observed in these pathways (data not shown). These results indicate that different enzymes are responsible for N-demethylations and C-8 hydroxylation.

The enzyme activities in adult rats were compared with those in immature rats (3 weeks old) (Fig. 3). Adult (male) rats showed higher activities of N-7 demethylation (theophylline formation) than immature rats ($P < 0.01$). Furthermore, adult (DA male) rats showed higher activities of N-1 and N-3 demethylations (theobromine formation and paraxanthine formation) than immature rats ($P < 0.05$). C-8 Hydroxylation (1,3,7-trimethyluric acid forma-

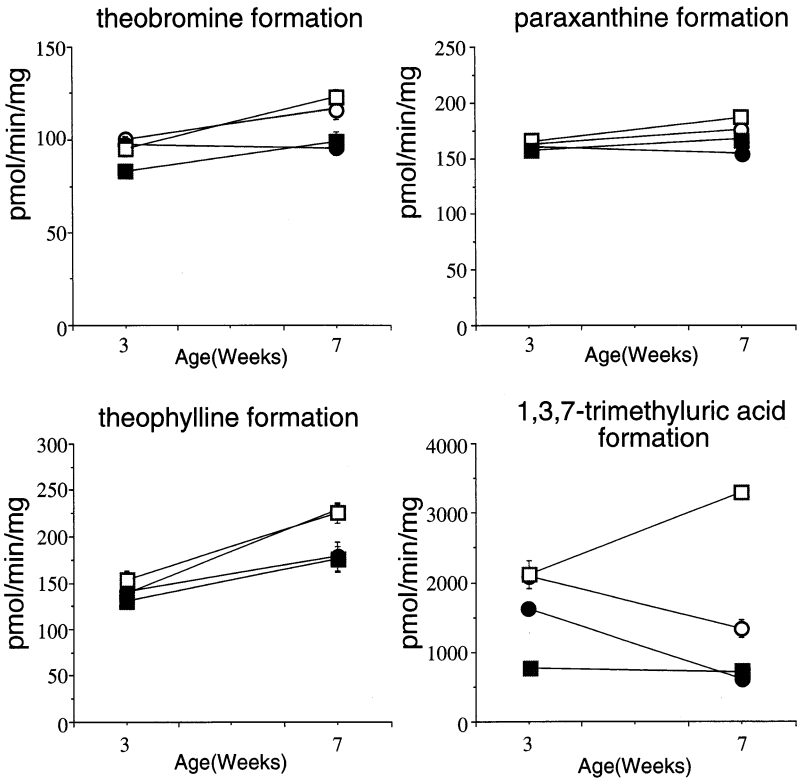

FIG. 3. Age-associated alterations in caffeine metabolism in rat liver microsomes. Caffeine metabolism in 3- and 7-week-old rat liver microsomes from Wistar male (○), Wistar female (●), DA male (□), and DA female (■) rats was determined. Formation of theobromine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid indicates N-1 demethylation, N-3 demethylation, N-7 demethylation, and C-8 hydroxylation. Incubation was carried out at 37° for 10 min with 10 mM of caffeine. Other experimental details are described under Materials and Methods. Data represent means ± SEM of four animals.

tion) differed considerably depending on the strain, sex, and age of the rats: adult Wistar rats showed lower C-8 hydroxylase activities than immature rats ($P < 0.01$); in contrast, adult DA male rats showed higher activities than immature rats ($P < 0.01$). Immature male DA and Wistar rats, however, showed similar activity levels of C-8 hydroxylation.

Table 1 shows the kinetic parameters of caffeine metabolism in adult male rat liver microsomes. *N*-Demethylase and C-8 hydroxylase (1,3,7-trimethyluric acid formation) activities were assayed at substrate concentrations of 0.5 to 32 mM. Both strains showed similar K_m and V_{max} values of N-demethylation. In the C-8 hydroxylation, both strains also showed similar K_m values, but the V_{max} values in male DA rats were about 5-fold higher than those of male Wistar rats. We presume that adult male DA rats have high levels of P450 isoform(s) that are responsible for the C-8 hydroxylation. Total concentrations of P450 have been shown to be similar in the DA and other rat strains [24].

The results of the addition of TAO in the incubation mixture for caffeine metabolism are presented in Fig. 4. TAO, which is a macrolide antibiotic that is metabolized to an unstable intermediate, causes an inactivation of cytochrome P450 by forming a stable complex with the iron of the hemoporphyrin [25, 26]. As this reaction with TAO is known to be specific to CYP3A isoenzymes [26], TAO was used for a provisional identification of the enzymes responsible for the N-demethylations and C-8 hydroxylation (1,3,7-trimethyluric acid formation) of caffeine. TAO effectively reduced hepatic microsomal C-8 hydroxylation in a concentration-dependent manner, but did not affect the N-demethylations.

The effects of anti-rat CYP3A2 antibody on caffeine N-demethylations and C-8 hydroxylation (1,3,7-trimethyl-

TABLE 1. Kinetic parameters of caffeine metabolism in rat liver microsomes

Metabolite	Strain/Sex	K_m (mM)	V_{max} (pmol/min/mg)
Theobromine	WM	8.89 ± 2.80	191.01 ± 14.80
	DM	11.24 ± 2.14	207.76 ± 24.21
Paraxanthine	WM	34.40 ± 5.02	654.87 ± 102.10
	DM	28.14 ± 6.13	635.54 ± 45.39
Theophylline	WM	13.17 ± 1.13	393.57 ± 62.20
	DM	15.60 ± 2.35	470.17 ± 77.14
1,3,7-Trimethyluric acid	WM	11.26 ± 0.91	1960.82 ± 221.14
	DM	13.57 ± 1.52	9459.10 ± 328.84*

Data are means ± SEM of three determinations. Abbreviations: WM, Wistar male; and DM, DA male.
*$P < 0.01$, compared with WM.

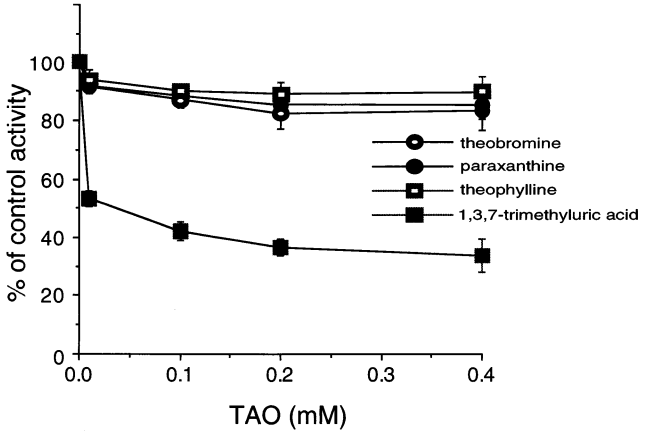

FIG. 4. Effect of TAO (CYP3A inhibitor) on caffeine metabolism in male DA rat liver microsomes. Formation of theobromine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid from caffeine were determined using liver microsomes from three rats. Control activities for the formations of these metabolites were, respectively, 125 ± 12, 222 ± 18, 225 ± 42, and 3445 ± 220 pmol/min/mg. Incubation mixtures containing TAO were preincubated in the presence of the NADPH-generating system at 37° for 15 min, and the reaction of the caffeine metabolism was initiated by the addition of 10 mM of substrate. Activities are expressed as a percentage of control activities. Other experimental details are described under Materials and Methods. Data are means ± SEM of three determinations.

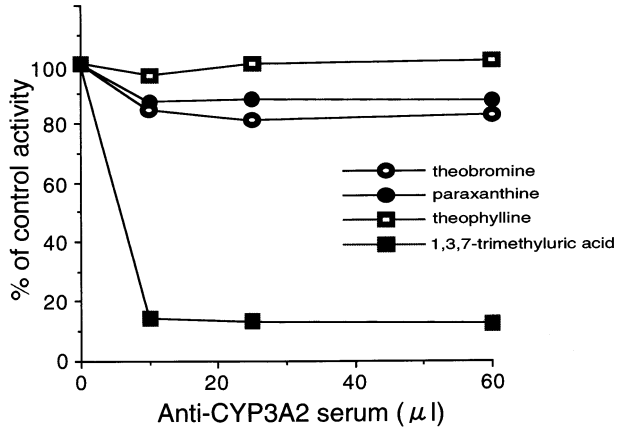

FIG. 5. Effect of anti-rat CYP3A2 antibody on caffeine metabolism in male DA rat liver microsomes. Formation of theobromine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid indicates N-1 demethylation, N-3 demethylation, N-7 demethylation, and C-8 hydroxylation. Immunoinhibition studies with the anti-rat CYP3A2 were carried out by preincubating antiserum with microsomes (0.1 mg of protein) at room temperature for 30 min prior to the measurement of caffeine metabolite formation. Control incubations contained an equivalent amount of preimmune rabbit serum proteins. Activities are expressed as a percentage of control activities. Control activities in the presence of preimmune serum were 118 ± 16 pmol/min/mg for theobromine formation, 175 ± 11 pmol/min/mg for paraxanthine formation, 232 ± 16 pmol/min/mg for theophylline formation, and 3320 ± 12 pmol/min/mg for 1,3,7-trimethyluric acid formation (means ± SEM for three determinations). The figure represents a typical result out of three immunoinhibition studies using microsomes from three male DA rats. Other experimental details are described under Materials and Methods.

uric acid formation) in male DA liver microsomes are shown in Fig. 5. Similar results were obtained using liver microsomes from male Wistar rats (not shown). An anti-rat CYP3A2 caused > 80% inhibition of the C-8 hydroxylation, but did not inhibit the N-demethylations. Therefore, we presumed that the rat liver microsomal C-8 hydroxylation is mediated largely by an isoform(s) of the CYP3A subfamily. We can also assume from this experiment that CYP3A is solely responsible for the high C-8 hydroxylase activity in DA rats.

Figure 6 shows the effect of treating male Wistar rats with the CYP1A inducer 3-methylcholanthrene and the CYP3A inducers phenobarbital (also an inducer of CYP2B), dexamethasone, and pregnenolone 16α-carbonitrile [27, 28] on the N-demethylations and C-8 hydroxylation (1,3,7-trimethyluric acid formation) by liver microsomes. 3-Methylcholanthrene treatment significantly raised the activity levels of N-1 and N-3 demethylations and C-8 hydroxylation. Phenobarbital treatment significantly raised the activity levels of all pathways. Dexamethasone and pregnenolone 16α-carbonitrile treatment significantly raised activity levels of C-8 hydroxylation only. Similar results were obtained using liver microsomes from DA rats (data not shown). We confirmed that the treatment of rats with CYP3A inducers causes a marked increase in C-8 hydroxylase activity. Because CYP3A antibody inhibits and the inducers of CYP3A enhance C-8 hydroxylase activity, it was concluded that CYP3A is capable of catalyzing C-8 hydroxylation of caffeine in rat liver microsomes.

Figure 7 shows the results of the immunoblotting analysis using anti CYP3A2 antiserum. Both strains of rats showed two bands with different staining intensities at a molecular mass of about 51 kDa. The upper band (a) representing CYP3A2 was induced markedly by pregnenolone 16α-carbonitrile. The other band (b) with lower molecular weight appeared in liver microsomes from Wistar and DA rats treated with pregnenolone 16α-carbonitrile. We could not assign this band at this time to any CYP isoforms. The CYP3A2 level of the male DA rat was higher than that of the male Wistar rat. This corresponds well with the high activity of C-8 hydroxylase (1,3,7-trimethyluric acid formation) in male DA rats. In addition, there was a sex difference (male > female) in the levels of CYP3A2 expression in both strains of rats.

A recent publication has reported that in humans liver microsomal C-8 hydroxylation (1,3,7-trimethyluric acid formation) is inhibited approximately 80% by an anti-CYP3A antibody [29] and is correlated with the immunoreactive CYP3A content [14]. We found that rat liver microsomal C-8 hydroxylation is also mediated largely by an isoform(s) of the CYP3A subfamily. In addition, C-8 hydroxylation is characteristically induced by phenobarbital, dexamethasone, or pregnenolone 16α-carbonitrile, known as CYP3A inducers.

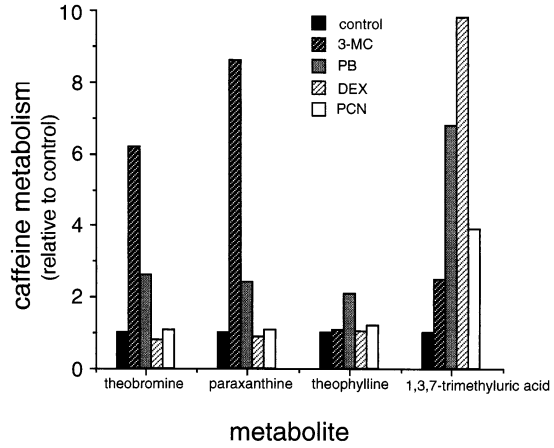

FIG. 6. Effect of P450 inducers on the N-demethylations and C-8 hydroxylation of caffeine in liver microsomes from male Wistar rats. Formation of theobromine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid indicates N-1 demethylation, N-3 demethylation, N-7 demethylation, and C-8 hydroxylation. Activities of these reactions in control Wistar rats were 118 ± 24, 176 ± 13, 223 ± 16, and 1422 ± 18 pmol/min/mg, respectively. Rats were treated with 3-methylcholanthrene (3-MC) (40 mg/kg/day for 3 days), phenobarbital (PB) (80 mg/kg/day for 3 days), dexamethasone (DEX) (100 mg/kg/day for 3 days), and pregnenolone 16α-carbonitrile (PCN) (25 mg/kg/day for 3 days). Other experimental details are described under Materials and Methods. Data are the means of three determinations.

CYP3A2 is developmentally regulated as the male-specific form [30]. Activities of CYP3A2-catalyzed reactions are higher in adult male rats than in female rats. It has been suggested that CYP3A2 is suppressed in adult female rats by their continuous pituitary growth hormone (GH) secretion profile, but that its expression is allowed in males by their pulsatile pattern of GH release [31]. As the CYP3A-mediated C-8 hydroxylation (1,3,7-trimethyluric acid formation) shows in Fig. 3, the pattern of CYP3A expression in male DA rats may be different from that in male Wistar rats. Our preliminary study on testosterone 6β-hydroxylation, a CYP3A-dependent reaction, has shown a similar strain difference between DA and Wistar rats [32].

In conclusion, our study has revealed that caffeine C-8 hydroxylation is mediated by CYP3A in rats and that the male DA rats, which are deficient in CYP2D, demonstrated high activity of CYP3A-mediated C-8 hydroxylase. If the high expression of CYP3A were always found in association with the low expression of CYP2D, the implications are manifold: to name a few, as in the case of DA rats, an extensive metabolizer of caffeine may be a poor metabolizer of debrisoquine, while the high expression of CYP3A may also be compensating for the poor/weak metabolism of a common substrate of CYP2D in individuals with a reduced expression of CYP2D. CYP2D is expressed even less in female DA rats, but the expression levels of CYP3A and its activities in female DA rats are as low as in female Wistar rats. This may indicate either that CYP3A is not compensating for the reduced expression of CYP2D, or, alternatively, the common physiological substrate for CYP2D and CYP3A exists only in male and not in female rats. Further studies are needed to verify these implications.

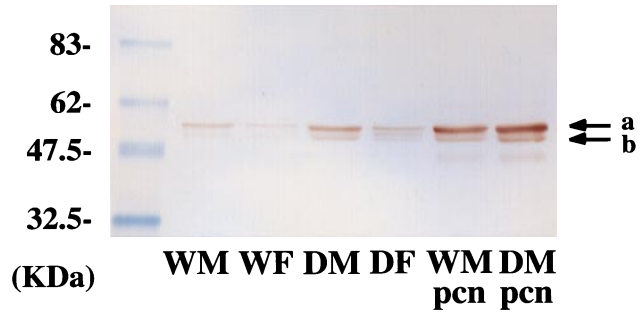

FIG. 7. Western blot analysis of liver microsomes taken from untreated or PCN-treated adult Wistar and DA rats using anti-P450 3A2 antiserum. Microsomal proteins (10 μg) were separated by SDS–PAGE and probed with anti-rat P450 3A2. Abbreviations: WM, Wistar male; WF, Wistar female; DM, DA male; DF, DA female; WM pcn, pregnenolone 16α-carbonitrile-treated Wistar male; and DM pcn, pregnenolone 16α-carbonitrile-treated DA male. Key: (a) upper band (CYP3A2); and (b) lower band (unknown protein related to CYP3A). Other experimental details are described under "Materials and Methods."

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