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Strain, Sex and Developmental Profiles of Cocaine Metabolizing Enzymes in Mice

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LEIBMAN, D., A. SMOLEN AND T. N. SMOLEN. *Strain, sex and developmental profiles of cocaine metabolizing enzymes in mice.* PHARMACOL BIOCHEM BEHAV **37**(1) 161–165, 1990.—Cocaine is a potent hepatotoxin in laboratory mice, although the cocaine-induced hepatotoxicity (CIH) is due to the action of a metabolite of cocaine. Cocaine can be hydrolyzed by serum cholinesterase (ChE) to inactive products, or be oxidized by hepatic cytochrome P-450 and FAD-containing monooxygenase (FADM). The oxidative pathway is thought to be responsible for production of the hepatotoxic metabolite of cocaine, presumably norcocaine nitroxide. Female mice are much more resistant to CIH than males of the same strain. We have found that immature male mice are as resistant as females to the development of CIH. Males did not show any CIH until the onset of puberty (30 days of age), indicating that the development of CIH in males was under hormonal control. To determine if the major cocaine-metabolizing enzymes were responsible for the regulation of CIH, we measured the activities of ChE, cocaine N-demethylation (CND) and FADM as a function of sex in C57BL/6Ibg and DBA/2Ibg mice 20–21, 30 ± 1 and 65 ± 5 days of age. There was a significant sex difference in ChE activity (females higher than males) but no effect of age. Cocaine N-demethylation increased in both males and females with age, but there was no consistent sex difference. Activity of FADM declined in males as a function of age, but remained constant in females. The lack of a consistent correlation between enzyme activities and sex-, strain-, and age-dependent differences in susceptibility to CIH, do not support a regulatory role for ChE, CND or FADM in mediating the hepatotoxic response.

Cocaine Hepatotoxicity Mice Development Cholinesterase Cocaine N-demethylase FAD-monooxygenase

COCAINE is a potent hepatotoxin in laboratory mice (8, 15, 26), although the cocaine-induced hepatotoxicity (CIH) is due to the action of a metabolite of cocaine, rather than cocaine itself (8,9). The majority of cocaine metabolism (90% or more) involves ester hydrolysis of O-carboxyphenyl and O-methyl groups on cocaine to ecgonine methyl ester and benzoyl ecgonine, respectively. The production of ecgonine methyl ester is catalyzed by serum cholinesterase and nonspecific tissue esterases (28,29), whereas benzoyl ecgonine is produced by spontaneous hydrolysis (28). None of the hydrolytic products when administered systemically have effects on the central nervous system, and none are capable of producing CIH (22, 29, 31). The alternative, oxidative pathway of cocaine metabolism, catalyzed by hepatic cytochrome P-450 and FAD-containing monooxygenase (FADM), is thought to be responsible for production of the hepatotoxic metabolite of cocaine (17, 19, 31). The first product of this pathway, norcocaine, is produced by N-demethylation of cocaine. Norcocaine is active in the central nervous system, and has been found in brain following peripheral administration of cocaine (12,21). Norcocaine can be metabolized further, and most investigators now believe that norcocaine nitroxide is ultimately responsible for the hepatotoxicity elicited by cocaine (19, 24, 25).

Mice are the species most susceptible to CIH, however, significant sex, age and strain differences to the hepatotoxic response have been reported. Females of all inbred strains examined are much more resistant to CIH than males of the same strain (2, 27, 32). Among the strains we have tested, male C57BL/6 and BALB/cBy are relatively resistant to CIH, whereas C3H and A strains are relatively susceptible to CIH (2,27). Recently, we reported that immature males of five inbred strains: A, BALB/cBy, C3H, C57BL/6 and DBA/2, were as resistant as females to the development of CIH. Males did not show any CIH until the onset of puberty (30 days of age), indicating that the development of CIH in males was under hormonal control (27).

Metabolic activation of cocaine is necessary for toxicity. The marked strain, sex and developmental differences in CIH suggest that one or more of the major enzymes involved in cocaine metabolism may regulate the differential expression of CIH. Regulation could result from high levels of detoxifying enzymes preventing the shunting of cocaine into the toxin-producing

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pathway, or by increased activity of oxidative enzymes in CIH-susceptible mice. In this paper we report our studies of strain, sex and developmental profiles of the major enzymes involved in cocaine metabolism in mice: plasma cholinesterase, microsomal cocaine N-demethylase and microsomal FAD-monooxygenase.

METHOD

Animals

Male and female C57BL/6Ibg and DBA/2Ibg mice 20–21, 30 ± 1 and 65 ± 5 days of age were used in these studies. Mice were born and raised at the Institute for Behavioral Genetics, maintained on a 12-hour light cycle (lights on 0700–1900) and allowed free access to food (Wayne Lab Blox) and water. All procedures reported in this paper were reviewed and approved by the University of Colorado Animal Care and Use Committee as being consistent with USPHS standards of humane care and treatment of laboratory animals.

Blood Collection

Blood for cholinesterase assays was collected from the retro-orbital sinus. Animals were anesthetized with pentobarbital (60 mg/kg) prior to blood collection. Anesthesia did not affect subsequent analyses. Blood was collected into two heparinized microhematocrit tubes (approximately 150 μl). Plasma was prepared at room temperature by centrifuging the tubes in a table top IEC clinical centrifuge fitted with a hematocrit rotor for 10 min at the maximum setting (approximately 7150 rpm, $5125 \times g$). This procedure results in plasma devoid of any detectable hemolysis as indicated by measurement of hemoglobin (10).

Determination of Cholinesterase Activity

Cholinesterase activity (14), dibucaine number (13) and fluoride number (11) were determined in plasma. Plasma was diluted 1:100 in 100 mM sodium phosphate, pH 7.4 and warmed to 25°C. Diluted plasma (1.5 ml) was added to 1.5 ml of 50 μM benzoylcholine chloride in 100 mM sodium phosphate, pH 7.4 (prewarmed to 25°C). The determination of dibucaine number was measured by adding 10 μM dibucaine to the reaction mixture. Fluoride number was measured by adding 50 μM NaF to the reaction mixture. Dibucaine and fluoride numbers were determined in adult males only. Enzyme activity was monitored using a Gilford 2400 spectrophotometer by measuring the decrease in absorbance at 240 nm and 25°C due to hydrolysis of benzoylcholine, and calculated from the molar extinction coefficient, 6700 $M^{-1} \cdot cm^{-1}$

Preparation of Hepatic Microsomes

Mice were killed by cervical dislocation and the hepatic microsomes isolated according to the method of Chung and co-workers (4). All procedures were carried out at 0–4°C. After removal of the gall bladder, the livers were excised, rinsed in 0.25 M sucrose, blotted dry, weighed and homogenized in 6 volumes of 1.15% KCl/0.01 M sodium phosphate, pH 7.4. The homogenate was centrifuged at $10{,}000 \times g$ for 15 min. The resulting supernatant was centrifuged at $105{,}000 \times g$ for 1 hr. The microsomal pellet was resuspended in 4 ml of buffer and centrifuged as above. These washed microsomes were resuspended in 50 mM potassium phosphate buffer, pH 7.4, to yield a protein concentration of approximately 15 mg/ml, and used immediately for analysis. Using marker enzymes for cytosol (alcohol dehydrogenase), mitochondria (monoamine oxidase) and microsomes (NADPH cytochrome c reductase), the washed microsomes were found to be devoid of cytosolic or mitochondrial contamination. Protein concentrations were measured by the Lowry method (20) using bovine serum albumin as standard.

N-Demethylation of Cocaine by Hepatic Microsomes

The N-demethylation of cocaine by hepatic microsomes (primarily cocaine N-demethylase activity) was determined by measuring the rate of production of formaldehyde using a modification (15) of the method of Nash (23). The reaction mixture contained an NADPH generating system (2.5 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, 2 mM AMP, 15 mM KCl and 10 mM $MgCl_2$), 3 mM NADPH, 50 mM HEPES buffer, pH 7.5, 5 mM cocaine-HCl as substrate, and hepatic microsomes (0.2 to 0.3 mg protein) in a total reaction volume of 0.5 ml. Samples were incubated for 15 min at 37°C in a shaking water bath. The reaction was quenched by the addition of 100 μl of 25% $ZnSO_4$ and 100 μl of saturated $Ba(OH)_2$. The tubes were then centrifuged at $1{,}500 \times g$ for 10 min to remove the precipitated proteins. The resulting supernatant (0.5 ml) was mixed with 0.2 ml of Nash reagent (7.5 g ammonium acetate and 0.1 M acetyl acetone in 25 ml of distilled water), incubated at 60°C for 10 min and the absorbance was read against a water blank at 412 nm. Control experiments were run in the absence of NADPH. The amount of formaldehyde formed was calculated from the molar extinction coefficient of the formaldehyde complex, 8000 $M^{-1} \cdot cm^{-1}$. Results are expressed as nmoles formaldehyde formed/mg protein/min.

Determination of FAD-Monooxygenase Activity in Hepatic Microsomes

FADM activity was measured by a modification (33) of the method of Cashman and Hanzlik (3). The reaction mixture included an NADPH regenerating system (0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate and 0.5 units/ml glucose-6-phosphate dehydrogenase), 3 mM *n*-octylamine hydrochloride (10 μl of 150 mM aqueous solution), 1 mM thiobenzamide as substrate, hepatic microsomes (25 μl, yielding 0.5 to 0.8 mg microsomal protein/ml of reaction mixture) and 150 mM potassium phosphate buffer, pH 8.4, containing 1 mM EDTA, 2.5 mM $MgCl_2$ and 1% Triton X-100 in a total volume of 0.5 ml. Cuvettes were warmed to 35°C and monitored until a stable baseline was obtained. Reactions were initiated by the addition of thiobenzamide (20 μl of a 25 mM solution in acetonitrile) to the sample curvette. Blank curvettes contained 20 μl of acetonitrile in place of thiobenzamide solution. Absorbance changes were recorded and reaction rates were determined using the molar extinction coefficient of thiobenzamide S-oxide, 2930 $M^{-1} \cdot cm^{-1}$ at 370 nm. Results are expressed as nmoles thoibenzamide S-oxide formed/mg protein/min. Thiobenzamide S-oxidation at pH 8.4 in the presence of *n*-octylamine is specific for FADM activity in microsomes (33).

Statistical Analysis

Data were analyzed by analysis of variance using strain, sex and age as between-subjects factors. Differences in individual sample means were detected using the Tukey B-post hoc test or *t*-test. A *p*-value of 0.05 was considered significant, and is the only level reported.

RESULTS

The developmental profile of ChE activity is shown in Fig. 1. There were main effects of strain and sex, $F(1,55) = 206$ and 44,

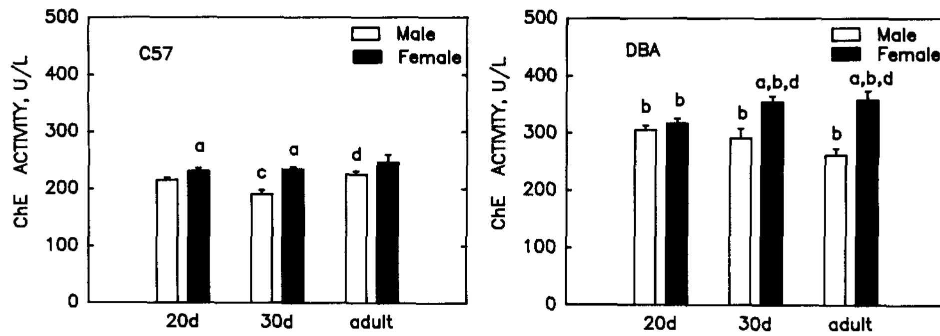

FIG. 1. Developmental profiles of plasma cholinesterase activity in male and female C57 and DBA mice. Values are mean ± SEM of 3–8 mice per age, sex and strain. a = Females significantly greater than males of the same strain and age. b = DBA mice significantly greater than C57BL mice of the same sex and age. c = Significantly different from 20-day-old mice of the same strain and sex. d = Significantly different from 30-day-old mice of the same strain and sex.

respectively, $p<0.05$, but not age, $F(2,55)=0.3$, NS. For all age groups and both sexes, DBA mice had significantly higher ChE activity than C57 mice. Activity of ChE in females was higher than the males, as has been reported previously (1). There were no strain differences in dibucaine numbers C57BL, 91.1 ± 1.0, DBA, 90.6 ± 0.2, or fluoride numbers C57BL, 21.5 ± 1.9, DBA, 15.7 ± 2.9 (mean ± SEM).

The developmental profile of cocaine N-demethylation (CND) by liver microsomes is shown in Fig. 2. There were significant main effects of strain and age, $F(1,56) = 105$ and 54, respectively, $p<0.05$, but not sex, $F(1,56) = 0.29$, NS. Cocaine N-demethylation in microsomes increased in both strains and sexes from 20 to 30 days of age. Adult levels were unchanged or lowered (C57BL males only) from the 30-day-old levels. The CND activity of the adult and 30-day-old C57BL mice was higher (not significant for adult males) than the CND activity of the DBA mice.

The development of FADM activity is shown in Fig. 3. There were significant main effects of strain, sex, $F(1,51) = 18$ and 68, $p<0.05$, and age, $F(2,51) = 4.3$, $p<0.05$. In contrast to CND activity, the effect of sex on FADM activity was very pronounced. For both strains the sexually immature males and females had the same level of the enzyme activity, but a significant difference in FADM activity was found at 30 days of age. The onset of puberty led to decreasing levels of FADM activity in male mice of both strains, although it was more pronounced in the C57BL mice. Female DBA mice showed an increase of FADM activity after puberty, but the magnitude was not statistically significant. The difference in FADM between strains was relatively small and did not show any clear pattern.

DISCUSSION

The striking difference in development of CIH between male and female mice indicates that the ultimate hepatotoxin is produced by a sexually dimorphic metabolic pathway. The enzyme responsible for production of that hepatotoxin is still not known. Our previous studies have shown that this pathway is also under developmental control, since prepubescent males do not have a hepatotoxic response when administered cocaine. Sex, strain and developmental differences are possible for any of the enzyme systems along the multistep oxidative pathway leading to the hepatotoxic metabolite of cocaine. For a pathway to be responsible for production of a toxic cocaine metabolite, it should be more active in adult males than adult females, and more active in adult males than in 30- or 20-day-old males. Similarly, DBA/2 mice, which are more susceptible to CIH than C57BL/6 mice, would be expected to have higher activity of an enzyme responsible for production of a hepatotoxin. Alternatively, if the difference in susceptibility to CIH is regulated by high activity of detoxifying enzymes, we would expect lower levels in groups susceptible to

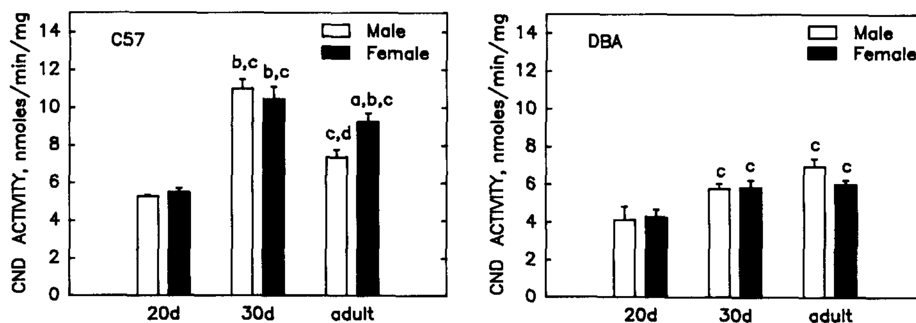

FIG. 2. Developmental profiles of cocaine N-demethylase activity in male and female C57 and DBA mice. Values are mean ± SEM of 3–8 mice per age, sex and strain. a = C57BL females significantly greater than C57BL males. b = C57BL mice significantly greater than DBA mice of the same sex and age. c = Significantly different from 20-day-old mice of the same strain and sex. d = Significantly different from 30-day-old mice of the same strain and sex.

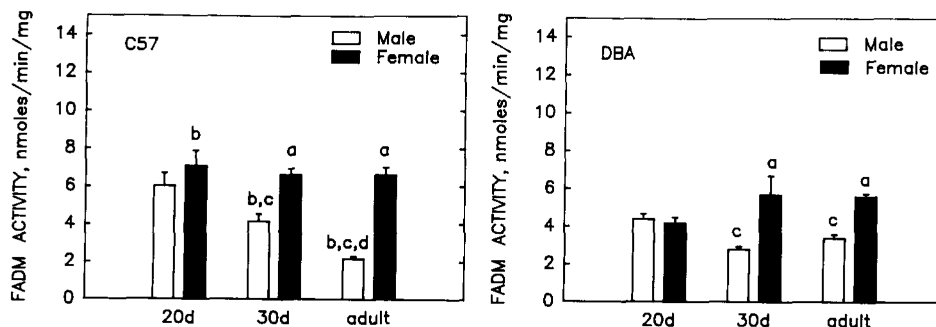

FIG. 3. Developmental profiles of FAD-monooxygenase activity in male and female C57 and DBA mice. Values are mean ± SEM of 3–8 mice per age, sex and strain. a = Females significantly greater than males of the same strain and age. b = C57BL mice significantly greater than DBA mice of the same sex and age. c = Significantly different from 20-day-old mice of the same strain and sex. d = Significantly different from 30-day-old mice of the same strain and sex.

CIH, especially adult males. By combining genetic, developmental and sexual differences in production of CIH it may be possible to determine the enzyme system responsible for the production of the hepatotoxic metabolite of cocaine.

Serum cholinesterase represents the major, detoxifying pathway of cocaine metabolism. This enzyme could be involved in regulating sex, strain and age differences in response to cocaine by competing for cocaine with enzymes of the toxin-producing oxidative pathway. Experimentally, pretreatment of mice with the potent esterase inhibitor diazinon was found to increase CIH (32) and it has been suggested that individuals with low ChE activity may be susceptible to CIH (19). Our results, however, suggest that under normal physiological conditions ChE has little regulatory effect on hepatotoxicity. The comparison between the developmental profile of ChE activity (Fig. 3) and the developmental profile of cocaine hepatotoxicity in the same strains (27) shows that there is a dramatic increase in CIH between 20-day-old and adult male mice, without a corresponding change in plasma ChE activity. Moreover, both strains appear to have the same ChE phenotype (and catalytic properties) since there was no difference in activity level, dibucaine number or fluoride number.

The first step in the pathway of production of a toxic metabolite of cocaine requires the N-demethylation of cocaine to norcocoaine (9,31). Norcocaine may be produced by two alternate pathways: direct N-demethylation by cytochrome P-450 (cocaine N-demethylase), or sequentially by FADM and cytochrome P-450 via a cocaine N-oxide intermediate (17,19). The direct cytochrome P-450-mediated pathway accounts for approximately 85% of the total N-demethylation of cocaine [(17); present results] and the data in Fig. 2 represent primarily the direct pathway of norcocaine formation. It has been suggested that the species selective hepatotoxicity of cocaine is due to differences in the rate of formation of norcocaine (7). High levels of N-demethylase activity may be associated with increased liver damage following cocaine as indicated by studies in which CIH was increased in mice previously treated with the cytochrome P-450 inducer phenobarbital (8,15,31). Our data do not support that hypothesis, however. Cocaine N-demethylation does not appear to be modulated by sex hormones since there was no consistent sex difference in activity at any age in either strain [see also (32)]. Enzyme activity increased with age, but the pattern does not correlate with susceptibility to CIH. DBA males are more susceptible to CIH than C57BL males, yet the C57BL mice had higher CND activity than DBA mice (Fig. 2). Similarly, C57BL females are slightly more susceptible to CIH than DBA females (2,27), but CND activity in C57BL females was much higher than in DBA females.

The secondary pathway of norcocaine production is catalyzed sequentially by FADM and cytochrome P-450 (17). Activity of FADM is under control of sex hormones (6, 16, 32), and the developmental profile in mice has been studied by two other groups. Wirth and Thorgeirsson (34) reported that hepatic FADM activities in males and females paralleled each other until puberty, at which time the activities of female mice increased, while those of males remained at their low prepubescent levels. Kloss *et al.* (16), however, reported a decrease of FADM activity in males from the onset of puberty to adulthood, while FADM activities in females remained at their high prepubescent levels. Our results are in agreement with the later finding and support their conclusion that the sex differences in hepatic FADM activity appear to be mediated through the suppressive effect of testosterone.

We found that adult female mice had significantly higher FADM activity than adult males. This sex difference was absent in 20-day-old mice, and became apparent at the onset of puberty. However, our results suggest that FADM is not responsible for the sex difference in CIH response. The lower FADM activity in adult male mice compared to females or immature males should lead to decreased production of norcocaine by this route and, therefore, to decreased CIH, which is the opposite of what is observed in vivo. A protective role for FADM could be suggested from its developmental profile in males (high activity in nonsusceptible groups, especially apparent in C57BL mice); however, the data do not consistently support that hypothesis. For example, adult C57BL males are more resistant to CIH than DBA males, yet have lower FADM activity, and 30-day-old DBA males are more resistant to CIH than adults, yet their FADM activities do not differ. Moreover, FADM is postulated to be involved in the next step in hepatotoxin synthesis, catalzying the conversion of norcocaine to N-hydroxynorcocaine. Clearly, the low level of FADM in adult males is not consistent with a regulatory role for FADM in mediating CIH.

The toxic metabolite of cocaine is not known, although norcocaine nitroxide is generally regarded as the ultimate hepatotoxin. The mechanism by which it causes cellular damage remains speculative. It has been suggested that norcocaine nitroxide is involved in a "futile" oxidative cycling reaction with N-hydroxynorcocaine, catalyzed by FADM in one direction and cytochrome P-450 in the other (19) resulting in production of superoxide anion radicals, hydrogen peroxide, decreased reduced glutathione levels and, finally, increased lipid peroxidation. While there is supportive evidence for such a system in vivo and in vitro (18,25), results of two recent studies found that while cocaine caused changes in cellular thiols in vivo and in isolated rat and mouse hepatocytes,

lipid peroxidation was not a critical determinant of cocaine-induced toxicity (5,30). Alternatively, cocaine or an activated metabolite could bind to tissue macromolecules, disrupting their function. Evans and Harbison (9) found an activated metabolite (presumably) of cocaine bound to hepatic protein in vivo, although the identity of the bound species has not been determined.

The enzyme responsible for the production of the toxic metabolite of cocaine has not been identified, although our developmental data suggest that it is induced by androgens during a narrow age range. Each of the cocaine metabolizing enzymes measured in this study, serum cholinesterase, hepatic cocaine N-demethylase or hepatic FAD-containing monooxygenase, has been hypothesized to be involved in mediating the CIH response in mice. All are clearly involved in cocaine metabolism and participate to some extent in the hepatotoxic response. The lack of consistent correlation between enzyme activities and sex-, strain- and age-dependent differences in susceptibility to CIH do not support a regulatory role for any of them in mediating this response. It is our hypothesis that an androgen-induced enzyme, possibly a form of cytochrome P-450 is responsible for this regulation, and future studies will concentrate on this aspect of cocaine-induced hepatotoxicity.

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