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# SHORT COMMUNICATIONS

## Amine oxidase in mice—sex differences and developmental aspects

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The metabolism *in vivo* and *in vitro* of tertiary amine drugs and foreign compounds (xenobiotics) has been extensively studied over the years. Of the various pathways by which tertiary amines can be metabolized, the major route is via a cytochrome P-450-catalyzed oxidative *N*-dealkylation. However, with some tertiary amines, such as morphine and chlorpromazine, oxidation to the *N*-oxides is also observed. The enzyme systems catalyzing oxidative *N*-dealkylation and *N*-oxide formation of tertiary amines are very similar. Both enzymes are mono-oxygenases which are localized primarily in the hepatic microsomal fraction and both require NADPH and molecular oxygen for activity [1, 2]. The enzymes differ in that dealkylation is dependent on cytochrome P-450, whereas the terminal oxidase in *N*-oxide formation is a flavin-containing enzyme.

In contrast to the extensive literature which exists concerning the oxidative dealkylase enzyme system(s), much less is known about the other mixed function amine oxidases. Ziegler *et al.* [1, 3] have purified to homogeneity a pig liver microsomal mono-oxygenase which catalyzes the oxidation of a large number of lipid soluble secondary and tertiary amines to the corresponding hydroxyamines and amine oxides respectively. Although hepatic amine oxidase has been found in all vertebrates studied, including man, very little is known about the ontogenicity of this enzyme in the mouse liver or its relationship to other oxidative enzyme systems, such as the hepatic cytochrome P-450 mixed function mono-oxygenases. The present investigation was undertaken to determine the hepatic microsomal amine oxidase enzyme system in the developing liver of both male and female inbred mice. In addition, the effects of pretreatment with compounds, known to markedly induce the oxidative dealkylase system(s), on hepatic *N*,*N*-dimethylaniline (DMA) *N*-oxide formation (via amine oxidase) and DMA *N*-demethylase activity (cytochrome P-450 mediated) in the mouse liver, were also studied.

### MATERIALS AND METHODS

The inbred mouse strain, B6,* was obtained from National Institutes of Health, Animal Production Section. Mice were allowed food (Purina Lab Chow) and water *ad lib.* and were housed under conditions of controlled temperature and lighting.

DMA was obtained from Aldrich Chemical Co. (Milwaukee, WI) and redistilled prior to use, dissolved in equimolar amounts of HCl, and diluted with distilled water to the appropriate concentrations. The pH of the final solutions was adjusted to pH 2.0 with hydrochloric acid. Each solution was prepared daily as needed. 3-MC was purchased from Eastman Kodak Co. (Rochester, NY) and TCDD was a generous gift from Dr. Alan P. Poland, University of Rochester School of Medicine and Dentistry, to Dr. D. W. Nebert, NICHD, National Institutes of Health (Bethesda, MD). NADPH was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade and obtained from commercial sources.

*Treatment of animals.* Sexually matured mice were injected intraperitoneally with 3-MC in corn oil with a single dose of 80 mg $kg^{-1}$ body weight. Control animals received corn oil only. Animals were sacrificed 48 hr after the last injection. PB in 0.9% saline was administered intraperitoneally daily for 3 days at a dose of 75 mg $kg^{-1}$. Control animals received 0.9% saline only. All animals were sacrificed on day 4. TCDD was administered intraperitoneally in dioxane at a dose of 100 $\mu g\ kg^{-1}$. Control animals received dioxane only. Animals were sacrificed 48 hr after the last treatment. DMA was given intraperitoneally at various doses for 4 days and sacrificed 24 hr after the last dose.

*Tissue preparation.* All animals were killed by cervical dislocation. Immediately after exsanguination the livers were removed and placed in ice-cold 0.25 M KCl-phosphate buffer, pH 7.25. All subsequent operations were performed at 0°. The livers were minced, washed as free of hemoglobin as possible, and homogenized in 3 vol. of KCl-phosphate buffer in a motor driven Potter–Elvehjem type homogenizer with a Teflon pestle. The homogenate was centrifuged at 9000 *g* and the supernatant was decanted and centrifuged at 105,000 *g*, for 60 min. The microsomal pellet was resuspended in 0.25 M glycerol phosphate buffer, pH 7.25, and either used immediately or stored at −70° until needed. Results from control experiments showed no significant loss of amine oxidase activity upon storage of microsomes for up to 30 days at −70° in the glycerol phosphate buffer. Protein concentration was determined by the method of Lowry *et al.* [4] using bovine serum albumin as a standard.

*Enzyme assays.* DMA *N*-oxide formation (amine oxidase) and DMA *N*-demethylase activities were determined as previously reported [5] with slight modifications.

Incubations were performed in open 25-ml Erlenmeyer flasks at 37° in a Dubnoff metabolic shaker. Assay mixtures contained in a total volume of 5 ml the following: 0.1 M alanine (500 $\mu$moles)–0.025 M pyrophosphate (125 $\mu$moles) buffer, pH 8.4; NADPH, 10 $\mu$moles; microsomal protein, 5 mg; semicarbazide, 5 $\mu$moles; and *N*,*N*-dimethylaniline, 5 $\mu$moles.

After preincubation at 37° with shaking for 3 min, the reactions were initiated with the addition of either microsomes or DMA, and incubated with shaking for 10 min at 37°. The reactions were terminated by the addition of 0.6 ml of 1 M trichloroacetic acid, after which they were centrifuged at 1000 *g* for 10 min. DMA *N*-demethylase activity was determined by following the production of formaldehyde using the Nash procedure [6].

In order to assay for DMA *N*-oxide formation, a 3.6-ml aliquot of the deproteinized supernatant was adjusted to

* Abbreviations used are: B6, the inbred C57BL/6N mouse strain; DMA, *N*,*N*-dimethylaniline; 3-MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; NADPH, reduced pyridine nucleotide; PB, phenobarbital; and AHH, aryl hydrocarbon (benzo[a]pyrene) hydroxylase.

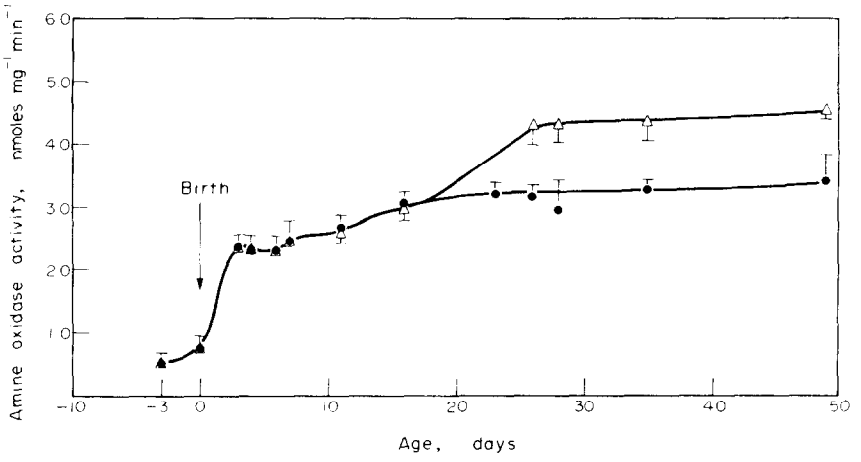


Fig. 1. Developmental curve for the amine oxidase in C57BL/6N mice. Key: (●——●) male mice; and (△——△) female mice. Each point is mean ± S. D. of six to eight mice.

pH 9–10 with 0.80 ml of 1 M $NaHCO_3$ and 0.24 ml of 6 M KOH and extracted three times with diethyl ether to remove excess DMA. After extraction, the aqueous solution was heated at 60° for 10 min to remove any dissolved ether. An aliquot (2.7 ml) of the aqueous solution containing the DMA *N*-oxide was adjusted to pH 2.5 with 0.24 ml of 3 M trichloroacetic acid, 0.6 ml of 1 M glycine buffer, pH 2.5, and 0.3 ml of 1 M $NaNO_2$. The solution was then heated at 60° for 6 min. DMA *N*-oxide formation was measured spectrophotometrically (420 nm) using the published mM extinction coefficient (8.2 $cm^{-1}$) of the *p*-nitrosodimethylaniline derivative [7].

Microsomal cytochrome P-450 content was estimated by its CO-binding difference spectrum as described by Omura and Sato [8], using an Aminco DW-2 spectrophotometer.

## RESULTS AND DISCUSSION

The development of DMA *N*-oxidase activity in maturing livers from both male and female B6 mice was determined and the developmental curve of enzymic activity is illustrated in Fig. 1. No attempt was made to determine the sex of the mice during the period between days −3, 0, 3, 4, 6 and 7; therefore, no comparison of DMA *N*-oxidase activity between the sexes was made at these ages. The activity of liver DMA *N*-oxidase at 3 days before birth was about 15 per cent of the maximal activity observed in the adult mice. A similar level of DMA *N*-oxidase activity was found at birth, but at 3 days of age this activity was about 50 per cent of the maximum activity found in the adult livers. At day 11 the sexes could be easily determined; however, there appeared to be no quantitative difference in DMA *N*-oxidase activity in livers from either male or female B6 mice at this age. There was a gradual increase in activity in both male and female livers with no apparent difference in specific activity until between day 16 and day 26. During this period, there was a marked increase in DMA *N*-oxidase activity (2.92 → 4.30 nmoles $mg^{-1}$ $min^{-1}$) in the livers from female B6 mice which reached a maximum level at day 26 and remained fairly constant at that level. However, in livers from male B6 mice, hepatic DMA *N*-oxidase activity reached a maximum level, which was 74 per cent of that observed in the female, at day 16 and remained fairly constant at that level at least until week 10 (data not shown in Fig. 1). DMA *N*-oxidase activity was also determined in the livers from nursing mother mice, and in these animals the *N*-oxidase activity was 4.66 nmoles $mg^{-1}$ $min^{-1}$. The developmental aspects of DMA *N*-oxidase in the mouse are considerably different from those observed for cytochrome P-450-dependent hydroxylations. For example, the basal AHH activity in liver is not detectable before birth in B6 mice, and at 3 days of age the AHH activity is less than 25 per cent of the adult level [9]. Similar observations for the development of AHH activity in the rat have also been made [10]. The age distribution of the DMA *N*-oxidase activity in liver, however, has so far only been done in rabbits [11] and rats [12, 13]. In the rabbit, DMA *N*-oxidase activity is fairly low at 3 days of age (20 per cent of the adult level) and then increases at a relatively steady rate until adulthood. However, in marked contrast to both the mouse and rabbit, the developmental curve of *N*-oxidase activity in rats shows two peaks of DMA *N*-oxidase activity, one at about 3 days of age and the other at about 27 days of age [13]. The peak activity at 3 days is about 50 per cent of the peak activity observed at day 27. Our results in the mice are in good agreement, except that we did not observe any decrease in *N*-oxidase activity between day 3 and day 27. In addition, after 30 days, sex differences appeared, and the *N*-oxidation of DMA in microsomes of female rats 60 days old was only 40–50 per cent compared with that of male rats [13]. Our results similarly showed strong sex differences in the *N*-oxidation of DMA. However, these differences appeared much earlier in the mouse (between day 16 and day 26) than they did in the rat (day 30); furthermore, our results showed that hepatic amine oxidase activity in the male mice was only 74 per cent of that in the female mice.

Polycyclic hydrocarbons, such as 3-MC, have been shown to induce cytochrome P-450-mediated mono-oxygenase system(s) in the liver and other tissues of certain inbred strains of mice but not in others. Increases in at

Table 1. Hepatic microsomal *N*-oxidation of DMA in female C57BL/6N (B6) mice after treatment *in vivo* with 3-MC, PB and TCDD*

| Treatment | DMA *N*-oxide formed (nmoles $mg^{-1}$ $min^{-1}$) |
|---|---|
| None | 3.97 ± 0.18 (6)† |
| 3-MC (80 mg $kg^{-1}$ $day^{-1}$ × 1) | 4.10 ± 0.08‡ (8) |
| PB (75 mg $kg^{-1}$ $day^{-1}$ × 3) | 2.89 ± 0.25§ (8) |
| TCDD (100 μg $kg^{-1}$ $day^{-1}$ × 1) | 4.45 ± 0.08‡ (4) |

* Values are expressed as means ± standard deviation. Condition of the given assay is given under Materials and Methods.

† Number of determinations.

‡ Not different from untreated: $P > 0.05$ (*t*-test).

§ Different from untreated: $P < 0.001$ (*t*-test).

Table 2. Hepatic microsomal *N*-oxidation, *N*-demethylation and P-450 concentration in female C57BL/6N (B6) mice pretreated with DMA*

| DMA dose (mg $kg^{-1}$ $day^{-1}$ for 4 days) | DMA *N*-oxide formed (nmoles $mg^{-1}$ $min^{-1}$) | DMA Formaldehyde formed (nmoles $mg^{-1}$ $min^{-1}$) | Cytochrome P-450 (nmoles $mg^{-1}$) |
|---|---|---|---|
| 0 | 3.73 ± 0.08 | 2.60 ± 0.15 | 0.516 ± 0.060 |
| 25 | 4.44 ± 0.14† | 2.79 ± 0.24† | 0.486 ± 0.072† |
| 50 | 4.30 ± 0.22‡ | 2.59 ± 0.22† | 0.543 ± 0.030† |
| 125 | 3.67 ± 0.24† | 2.31 ± 0.13† | 0.496 ± 0.047† |
| 250 | 4.14 ± 0.39† | 1.79 ± 0.29‡ | 0.271 ± 0.028‡ |

* Values are the means ± standard deviation of four to eight experiments. Conditions of the assays are given under Methods.

† Not different from 0 dose: $P > 0.05$ (*t*-test).

‡ Different from 0 dose: $P < 0.05$ (*t*-test).

least ten mono-oxygenase activities, including aryl hydrocarbon hydroxylase (AHH), *N*-hydroxylation, and *N*- and *O*-dealkylation reactions have been observed in liver microsomes of the "responsive" B6 mice that have been pretreated with 3-MC but not in the liver from similarly pretreated "nonresponsive" D2 mice [14]. Phenobarbital, a relatively nonspecific inducer of cytochrome P-450-dependent mono-oxygenase(s) activities, increases these activities, but to a lesser extent, in both strains of mice. Furthermore, the potent enzyme inducer TCDD induces AHH as well as *N*-hydroxylase activities in both the responsive and nonresponsive strains of mice to the same extent as seen in the responsive strains after 3-MC treatment [14, 15].

Table 1 shows the effect of pretreating female B6 mice with 3-MC, PB and TCDD on DMA *N*-oxidase activity in the liver microsomes. No significant increase is seen in the activity of DMA *N*-oxidase after these pretreatments. PB pretreatment decreases the *N*-oxidase activity slightly, whereas the *N*-dealkylation is increased 2-fold (data not shown). The failure, however, to observe any significant increase in amine oxidase activity even after pretreatment with TCDD, the most potent known inducer of cytochrome P-450-associated mono-oxygenase activities, reflects the refractiveness of amine oxidase toward enzyme induction.

The effect of DMA pretreatment *in vivo* on *N*-oxide formation and formaldehyde formation from DMA, as well as cytochrome P-450 concentration in liver microsomes from female B6 mice, is shown in Table 2. At low doses (25–125 mg $kg^{-1}$ for 4 days), DMA increased *N*-oxidase activity slightly, but had no effect on *N*-dealkylation or cytochrome P-450 content. At the highest dose (250 mg $kg^{-1}$ for 4 days), DMA significantly reduced the rate of DMA *N*-dealkylation and the microsomal concentration of cytochrome P-450, but had no effect on DMA *N*-oxide formation. On histological examination, the liver showed fat infiltration and single cell deaths after the highest dose of DMA but no fat infiltration or single cell deaths were observed at the lower doses. These data indicate that the two mono-oxygenases respond differently to the hepatotoxic effects of DMA, the cytochrome P-450-dependent mono-oxygenase being the more sensitive.

Our results indicate that developmentally the flavin-dependent mono-oxygenase (Ziegler's enzyme) is different from that of cytochrome P-450-dependent mono-oxygenase(s) in mice [9]. The most striking difference is that the activity of the amine oxidase is much higher shortly before and after birth than the activity of the cytochrome P-450-dependent oxidations. Since *N*-oxidation of secondary or tertiary amines may lead to the formation of potentially toxic metabolites and since many drugs contain secondary and tertiary amine groups, the newborn animal, and presumably also man in which significant levels of DMA *N*-oxidase activity have been found in both fetal and adult liver microsomes [16, 17], might be relatively more susceptible to these toxic effects than the adult.

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