Strain-dependent Differences in the Metabolism of 3-Methylcholanthrene by Maternal, Placental, and Fetal Tissues of C57BL/6J and DBA/2J Mice

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ABSTRACT

C57BL/6J (B6) and DBA/2J (D2) mice have different susceptibilities to developmental toxicity and transplacental carcinogenesis induced by in utero exposure to polycyclic aromatic hydrocarbons, which has been associated with polycyclic aromatic hydrocarbon metabolism and inducibility at the Ah locus. The distribution of total 3-methylcholanthrene (3-MC)-associated radioactivity in maternal, placental, and fetal tissues of β-naphthoflavone-pretreated pregnant B6 and D2 mice was determined up to 12 h after p.o. exposure to [6-14C]-3-MC (63 mg/kg, 20 μCi) on gestational day 17. 3-MC-associated radioactivity in maternal plasma was not significantly different in the two strains. However, D2 tissue homogenates had consistently higher levels of 3-MC-associated radioactivity, which included both bound and free parent compound and metabolites. Increased metabolism of 3-MC by B6 maternal liver was suggested by the induced levels of aryl hydrocarbon hydroxylase activity in that tissue and by the observation that levels of total radioactivity decreased more rapidly in B6 tissues than in D2 tissues. The D2 fetal lung, the target tissue for 3-MC-induced transplacental carcinogenesis, appeared to accumulate 3-MC-associated radioactivity for a longer period of time than either the D2 fetal liver or the B6 fetal tissues. This study suggests that the genetic differences in fetal susceptibility to the developmental toxicity and transplacental carcinogenesis of 3-MC may be related to the presystemic elimination of the compound from both maternal and fetal tissues.

INTRODUCTION

Experiments in rodents indicate that during late development the fetus is at greater risk than the adult to certain carcinogens [reviewed by Rice (1)]. Some transplacental carcinogens are direct acting and do not require metabolism to exert a carcinogenic effect. Another class of transplacental carcinogens, the PAHs, require enzyme-mediated metabolic conversion to reactive intermediates to induce carcinogenesis. The Ah locus controls inducibility by 3-MC and numerous other PAHs, of at least 20 monoxygenase activities, including AH. The wild mouse and the majority of inbred strains possess PAH "responiveness" due to induction of cytochrome P1-450 by PAH exposure. The prototype responsive strain is the C57BL/6J, or B6, mouse which possesses the Ah-b allele in the homozygous state. DBA/2J is the prototype nonresponsive strain, referred to as D2, which possesses the Ah-d allele in the homozygous state. Heterozygotes (Ah-b/Ah-d) from a B6 x D2 cross are responsive, indicating that P1-450 induction is inherited through an autosomal dominant mode (2).

Recent studies have indicated that inducibility of the Ah locus influences the transplacental carcinogenesis of 3-MC (3, 4). In these studies, backcross matings between F1 hybrid males (Ah-b/Ah-d) and inbred D2 females (Ah-d/Ah-d) resulted in noninducible pregnant mice (Ah-d/Ah-d) containing litters with a 1:1 ratio of inducible (Ah-b/Ah-d) and noninducible (Ah-d/Ah-d) fetuses. Dams were exposed p.o. to 3-MC on days 15–17 of pregnancy. Survival and tumor incidence were monitored in the offspring for up to 13 months of age and correlated with the genotype at the Ah locus. The noninducible offspring (Ah-d/Ah-d) had higher incidences of lung nodules, adenomas, and bronchiolar hyperplasia than inducible offspring (Ah-b/Ah-d). The latter had elevated rates of neonatal mortality and depressed body weights. These results indicate that when fetuses are exposed to 3-MC in the same noninducible maternal environment, genetic differences in fetal genotype at the Ah locus influence fetal susceptibility and toxicological outcome.

Studies in adult animals have indicated that the route of administration, in combination with allelic differences at the Ah locus, has a strong influence on the manifestation of PAH-induced toxicity [reviewed by Nebert (5)]. When the PAH is directly applied to the target tissue, the Ah-b allele is generally associated with increased susceptibility to toxicity at the site of administration. If BP is given topically, i.t., or i.p., the inducible mouse (Ah-b) is at greater risk than the noninducible mouse (Ah-d) for occurrence of lesions in the skin, the s.c. tissue, the lung, and the peritoneal cavity, respectively. The PAH is believed to be rapidly metabolized by inducible mice at the site of administration. In rodents, such tissues distant from the site receive relatively low levels of exposure. This has been referred to as "presystemic drug elimination" by Routeledge and Shand (6).

If the PAH is administered systemically, however, then the noninducible mouse is at increased risk in tissues distant from the site of administration. Exposure p.o. to BP results in higher incidences of hypoplastic bone marrow, leukemia, and lymphomas in noninducible than in inducible mice. With systemic exposure, the inducible mouse rapidly oxidizes, conjugates, and excretes the compound from the intestines and liver, while in the noninducible mouse more parent compound reaches remote tissues where it is slowly metabolized.

Recent studies have indicated that presystemic elimination plays an important role in the developmental toxicity of PAH. Legraverend et al. (7) performed backcrosses between homozygous and heterozygous mice to produce either inducible dams with a 1:1 ratio of inducible to noninducible embryos in the same litter or noninducible dams with litters containing the same ratio. With dietary exposure to BP during the period of major organogenesis, BP metabolism was greatly enhanced in the intestines and liver of inducible dams leading to reduction in the amount of parent compound/metabolites reaching the embryonic compartment. When the dam was inducible, no differences in prenatal toxicity between inducible and noninducible fetuses were observed. When the dam was noninducible, however, BP metabolism in the intestines and liver was negligible, and 10–20 times more compound reached the embryonic compartment. An unexpected finding was that noninducible embryos in these litters manifested increased prenatal toxicity.
compared to the inducible embryos. It was speculated that the inducible embryo was capable of more BP detoxification or that the noninducible embryo produced unique toxic BP metabolites. It was not possible to elucidate the metabolic profile of BP in inducible versus noninducible embryos in these litters since the entire litter was pooled to obtain enough embryonic tissue to carry out the pharmacokinetic analysis.

In the present study, the distribution of 3-MC-associated radioactivity was examined in maternal, placental, and fetal tissues of inbred B6 (inducible) and D2 (noninducible) mice after p.o. exposure on day 17 of pregnancy. The dams were pretreated on day 15 of pregnancy with β-naphthoflavone in order to maximize genetically regulated differences in AHH activity. The distribution of radioactivity in the maternal lung, liver, and placenta and in the fetal lung and liver of each strain was determined at various times after exposure to [6-14C]-3-MC. The purpose of this study was to ascertain whether pretreatment significantly increased AHH activity in maternal lung, maternal liver, and placenta, and in fetal lung and liver of each strain (i.e., 0.6% of body weight) using an 18-gauge, 1.5-inch curved stainless steel dosing tube (Perfektor; Popper and Sons, New Hyde Park, NY). The animals were sacrificed by CO2 asphyxiation at various times after 3-MC exposure. Maternal blood was collected by cardiac puncture into a heparinized syringe; the plasma was separated by centrifugation and stored at 0°C. Maternal lung, maternal liver, placenta, and fetal lung and liver were excised, rinsed in ice-cold 0.15 M KCl-0.25 M KH2PO4 buffer (pH 7.25, maternal liver; pH 7.4, other tissues), and stored in the same KCl-KH2PO4 buffer at ~80°C. Placentae, fetal lungs, and fetal livers were pooled by litter. Tissues were thawed at 4°C, rinsed in buffer, and homogenized in the KCl-KH2PO4 buffer of the appropriate pH using an Ultra-Turrax tissue homogenizer (Tekmar Co., Cincinnati, OH) at speed 6 for 5 s. Separate aliquots of the homogenate were saved for total protein and for AHH determinations. The remainder of the homogenate was extracted with 4 volumes of ethyl acetate (spectral grade; Fisher Scientific Co., Cincinnati, OH) to remove solvent-soluble parent compound and metabolites. The postextraction residue was analyzed for water-soluble and tissue-bound [6-14C]-3-MC-associated radioactivity.

Total Radioactivity Determinations. [6-14C]-3-MC-associated radioactivity in the entire tissue homogenate and the postextraction tissue residue was determined by liquid scintillation counting. Aliquots of tissue homogenate or postextraction residue were dissolved in Protosol (New England Nuclear) and decolorized with hydrogen peroxide prior to mixing with Aquasol II scintillation cocktail (New England Nuclear). Aliquots of maternal plasma were also analyzed for total radioactivity after direct addition to scintillation cocktail.

AHH Activity Determinations. AHH activity was determined by the method of Nebert (8) using the 12,000 x g (maternal lung, liver, and placenta) or the 750 x g (fetal lung and fetal liver) supernatants of the tissue homogenates. One unit of AHH activity was defined as that amount of enzyme producing the fluorescent equivalent of 1.0 nmol of authentic 3-hydroxybenzo(a)pyrene per min at 37°C. Sample fluorescence was measured on an Aminco-Bowman spectrophotofluorometer (Silver Springs, MD). The limit of detection of this method is approximately 0.1 pmol hydroxybenzo(a)pyrene/min/mg protein.

Protein Determinations. Protein content was determined by the micro-Lowry method, as modified by Schacterli and Pollack (9). Absorbance measurements were conducted on a Gilford model 2600 spectrophotometer (Oberlin, OH) with microprocessor-assisted linear regression analysis capability. Sample protein concentration was determined by comparison of the absorbance of the sample (A = 650) to a standard curve generated from bovine serum albumin reference samples (Sigma).

Statistics. Data were analyzed using the SAS Procedures GLM and TTEST (10, 11). Since individual animals were sacrificed at various times after exposure, time was used as an independent variable in a two-way analysis of variance for all data except AHH activity, which was analyzed using Student’s t test. If time-dependent data exhibited a significant strain by time interaction, pairwise comparisons at individual time points were conducted using the Tukey-Kramer multiple comparison test. Satterthwaite’s approximation of the degrees of freedom (12) was used when a variance ratio F test indicated homogeneity of variance. A type I error rate (α) of 0.05 was used to determine significance. Wherever appropriate, data are presented as mean ± SE.

RESULTS

β-Naphthoflavone pretreatment significantly increased AHH activity in all B6 tissues with the exception of the placenta (Table 2). In D2 animals, only AHH activity in the placenta was significantly increased over control levels after exposure to β-naphthoflavone (Table 2). When pretreated animals of the two strains were compared, B6 mice had significantly higher levels of AHH activity in the maternal lung, maternal liver, and

Table 1 Protocol for the treatment and sacrifice of pregnant B6 and D2 mice

<table>
<thead>
<tr>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gestational day 15: Treatment with corn oil</td>
<td>1. Gestational day 15: pretreatment with β-naphthoflavone</td>
</tr>
<tr>
<td>2. Gestational day 17: determination of basal levels of AHH activity in maternal lung, liver, and placenta and fetal lung and liver</td>
<td>2. Gestational day 17: administration of [6-14C]-3-MC, 63 mg/kg, 20 µCi, gavage</td>
</tr>
<tr>
<td>3. Sacrifice after 2, 4, 8, or 12 h and collection of maternal lung and liver placenta and fetal lung and liver</td>
<td>3. Sacrifice after 2, 4, 8, or 12 h and collection of maternal lung and liver placenta and fetal lung and liver</td>
</tr>
<tr>
<td>4. Homogenization of tissue</td>
<td>4. Homogenization of tissue</td>
</tr>
<tr>
<td>a. Determination of total radioactivity</td>
<td>a. Determination of total radioactivity</td>
</tr>
<tr>
<td>b. Determination of AHH activity</td>
<td>b. Determination of AHH activity</td>
</tr>
<tr>
<td>5. a. Extraction of homogenate with ethyl acetate</td>
<td>5. a. Extraction of homogenate with ethyl acetate</td>
</tr>
<tr>
<td>b. Determination of radioactivity in postextraction residue</td>
<td>b. Determination of radioactivity in postextraction residue</td>
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MATERNAL AND FETAL DISTRIBUTION OF 3-MC

Pretreated animals were given β-naphthoflavone (160 mg/kg i.p.) on day 15 of pregnancy, 48 h prior to sacrifice. Values are presented as pmol 3-hydroxybenzo(a)pyrene/min/mg protein (mean ± SE). N = 2 for each mean value.

<table>
<thead>
<tr>
<th>Biological Material</th>
<th>Control</th>
<th>Pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal lung</td>
<td>ND</td>
<td>30.51 ± 10.10</td>
</tr>
<tr>
<td>Maternal liver</td>
<td>43.44 ± 10.17</td>
<td>231.58 ± 54.84</td>
</tr>
<tr>
<td>Placenta</td>
<td>ND</td>
<td>7.25 ± 3.35</td>
</tr>
<tr>
<td>Fetal lung</td>
<td>ND</td>
<td>7.51 ± 1.84</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>0.46 ± 0.46</td>
<td>47.96 ± 19.37</td>
</tr>
</tbody>
</table>

* ND, not detectable.
* Pretreated > control animals of the same strain, t test, P < 0.05.
* Pretreated B6 > pretreated D2, t test, P < 0.05.

Table 2 Aeryl hydrocarbon hydroxylase activity in the tissues of control and β-naphthoflavone-pretreated B6 and D2 mice

Fig. 1. Total [6-14C]-3-MC-associated radioactivity in the maternal plasma and selected maternal and fetal tissues of B6 and D2 mice at various times after exposure to 3-MC. Pregnant mice were pretreated with β-naphthoflavone prior to receiving 3-MC by gavage (63 mg/kg, 20 μCi) on day 17 of pregnancy. Values for maternal plasma are presented as mean dpm/ml plasma. Values for total tissue radioactivity are presented as mean dpm/mg protein in the whole tissue homogenate. N = 2 for each time point. B6 (●); D2 (●). Significant overall strain-dependent differences (analysis of variance, P < 0.05) were obtained for maternal lung, placenta, fetal lung, and fetal liver. Asterisk, significant pairwise differences (P < 0.05) for individual time points.

DISCUSSION

Genetically determined differences in the metabolism of 3-MC by Ah-inducible and noninducible inbred mice have been postulated to be critical determinants in the prenatal toxicity which occurs after transplacental exposure to this and other PAHs. Recent studies have suggested that the genetic difference in PAH prenatal toxicity occurs as a result of presystemic elimination of the compound from the maternal system of the inducible animals, which reduces the amount of parent compound/metabolites reaching the fetal compartment. Direct detection in mouse fetal tissue of 3-MC and/or metabolic products by autoradiography after maternal administration of 3-MC has been reported, although individual metabolites were not quantitated (13). A comparison of the distribution of 3-MC-associated radioactivity in the maternal and fetal tissues of inducible and noninducible mice has not previously been reported. In this study, 3-MC-associated radioactivity was quantitated in the maternal, placental, and fetal tissues of B6 (inducible) and D2 (noninducible) mice at 2, 4, 8, and 12 h after p.o. exposure of β-naphthoflavone-pretreated pregnant animals to [6-14C]-3-MC.

Presystemic elimination after p.o. administration to pregnant animals requires that the compound in question be metabolized and/or eliminated from the maternal gastrointestinal tract or liver prior to reaching the plasma, other maternal tissues, or fetal lung; B6 placenta and fetal liver were not significantly increased over D2 tissues, although AHH activity in the B6 fetal liver was 10-fold higher than in D2 fetal liver (P = 0.058). [6-14C]-3-MC-associated radioactivity (dpm/ml plasma) in the maternal plasma of both strains of mice reached peak levels at 4 h after exposure and then declined to levels at 8 h which persisted relatively unchanged through the 12-h time point (Fig. 1). Total radioactivity in the maternal plasma of the two strains of mice was not significantly different although D2 animals tended to have higher levels than the B6 animals throughout the observation period. Total radioactivity (dpm/mg protein) in the tissue homogenates of maternal, placental, and fetal tissues was highest in the maternal liver and lung of both strains, followed by the placenta and fetal tissues (Fig. 1). Total radioactivity in the maternal liver did not differ significantly between the two strains but was strain dependent in the maternal lung, placenta, fetal lung, and fetal liver. For these tissues, each strain exhibited a characteristic profile of tissue radioactivity with respect to time after exposure. All of the B6 tissues reached peak levels of radioactivity at 2 h after exposure which were significantly higher than corresponding levels in D2 tissues and which subsequently declined to steady state levels at 4 h. D2 maternal lung, liver, placenta, and fetal liver continued to accumulate radioactivity for 4–8 h after exposure, with the maternal lung, placenta, and fetal liver radioactivity decreasing to steady state levels at 8 h. An unexpected finding was that the D2 fetal lung appeared to accumulate 3-MC-associated radioactivity for a longer period (2–8 h) than other D2 tissues, especially when compared to the D2 fetal liver (2–4 h). Peak levels of radioactivity in D2 maternal lung, placenta, fetal lung, and fetal liver were consistently higher than peak levels observed in corresponding B6 tissues.

Radioactivity in the postextraction residue of B6 and D2 fetal lung and liver was determined as an indication of the amount of 3-MC/metabolites bound to the fetal tissue or in the aqueous medium (Table 3). Although B6 fetal lung and liver had higher levels of nonextractable radioactivity at 2 h after exposure, D2 fetuses retained from 1.5 to 2.7 times more radioactivity during the 4–12-h period compared to B6 fetuses. For both tissues, accumulation of nonextractable [6-14C]-3-MC-associated radioactivity was strain dependent and exhibited a significant difference with regard to time.

the fetal compartment. In the present study evidence that pre-
systemic elimination was a determining factor in strain differ-
ences in the distribution of 3-MC and its metabolites between
β-naphthoflavone-pretreated B6 and D2 pregnant mice was
obtained. Initial levels of 3-MC-associated radioactivity were
equivalent in the plasma and livers of both the B6 and D2 mice,
indicating the absorption of the compound from the gastroin-
testinal tract was similar in both strains. Radioactivity in the
maternal plasma and liver disappeared more quickly in B6 than
in D2 maternal animals. Increased metabolism of 3-MC by the
B6 maternal liver was suggested by the induced levels of AHH
activity in that tissue. Consequently, after an equivalent p.o.
dose of labeled 3-MC, more radioactivity reached the D2 ma-
ternal plasma, maternal extrahepatic tissues, and subsequently
the D2 fetal compartment than in B6 mice.

Examination of individual fetal tissues indicated that in B6
animals, fetal lung and liver displayed a similar time course of
accumulation and disposition of radioactivity, with the fetal
liver having slightly higher levels than fetal lung 2 h after
exposure. In D2 animals, however, radioactivity initially ac-
culated in fetal liver and then shifted to fetal lung where
maximum tissue content was maintained up to 8 h. This pattern
of distribution can be understood in terms of fetal circulatory
routes and the levels of AHH activity in fetal liver and lung.
Foreign compounds which have crossed the placenta enter the
fetal circulation via the umbilical veins, which perfuse the fetal
liver (hepatic sinusoids) prior to reaching the fetal heart and
systemic circulation (14). In the B6 fetus, the induced levels of
hepatic AHH activity may result in rapid metabolism and
elimination of the compound from the fetal system such that
relatively less would reach the target tissue, the fetal lung. In
the D2 fetus, however, relatively little 3-MC metabolism would
occur in the liver due to low AHH activity, leading to increased
systemic circulation of parent compound and other metabolic
products derived from the maternal blood.

The occurrence of higher relative levels of radioactivity in the
D2 tissues suggests that slow elimination of parent compound
from these mice allows accumulation of intermediary metabo-
lites which are then available for further metabolism and sub-
sequent binding to tissue DNA and protein. The latter possi-
ability is suggested by the significant increase in radioactivity
associated with the postextraction residue of the D2 fetal lung
and liver.

These data suggest that the genetic differences in fetal sus-
cceptibility to 3-MC observed by York et al. (3, 4) may be related
to the role of presystemic elimination of the compound from
both the maternal and the fetal systems. In their experiments,
both inducible and noninducible fetuses in the same litter were
transplacentally exposed to 3-MC administered to D2 mothers.
Presystemic elimination of the parent compound from the
maternal system may be assumed to have been negligible re-
sulting in elevated and sustained exposure of the fetal compart-
ment to parent compound and intermediary metabolites in
maternal blood. Inducible offspring in the litter had elevated
rates of neonatal mortality and growth depression. The acute
toxicity response of inducible fetuses may have been a function
of their ability to rapidly metabolize and generate toxic inter-
mediates from transplacental products. Noninducible offspring
in the same litter did not demonstrate an acute toxicity response
shortly after birth but did not have significantly higher levels of
lung nodules, adenomas, and bronchiolar hyperplasias as adults
than inducible offspring. Based on results from the present
study, it can be postulated that the lack of lung lesions in
surviving inducible offspring may be due to first-pass metabo-
lism of the compound in the fetal liver after transplacental
exposure. For noninducible offspring, more parent compound
and intermediary metabolites may have been transported from
the fetal liver to the fetal lung where they accumulated, were
slowly metabolized and bound to lung tissue leading to in-
creased incidence of lung neoplasia.

The results of the present study and of York et al. suggest
that noninducible mothers are at greater risk for adverse preg-
ancy outcomes with prenatal exposure to 3-MC based on their
inability to presystemically eliminate the compound. The spe-
cific type of adverse pregnancy outcome manifested (i.e., neo-
natal death, growth impairment, childhood cancer) is influenced
by the fetal genotype, which in the case of the Ah locus deter-
mines the distribution and metabolism of the compound in the
fetal compartment.

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