CYP1A2 is not the primary enzyme responsible for 4-aminobiphenyl-induced hepatocarcinogenesis in mice

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4-Aminobiphenyl (4-ABP), a potent carcinogen in rodents (liver cancer) and human (bladder cancer), is found as an environmental contaminant and in tobacco smoke. Hemoglobin adducts and lung DNA adducts of 4-ABP are found in tobacco smokers. In vitro metabolism studies with human and rat liver microsomes have shown that CYP1A2 is primarily responsible for catalyzing N-hydroxylation, the initial step in the metabolic activation of 4-ABP. To determine whether this P450 is a rate limiting pathway for hepatocarcinogenesis, CYP1A2-null mice were analyzed at 16 months of age and were compared with wild-type mice in their response to 4-ABP using the neonatal mouse bioassay and two different doses of the carcinogen. Overall differences in incidences of hepatocellular adenoma, carcinoma and preneoplastic foci were not significant between either genotypes or 4-ABP doses used, whereas small, but significant, differences were found for specific types of foci. These results suggest that while CYP1A2 levels may not be rate limiting for 4-ABP metabolism to produce tumors and foci, it may modulate the induction process of some types of liver foci in either a positive or negative manner. In vitro studies using CYP1A2-null and wild-type mouse liver microsomes revealed that CYP1A2 is not the sole P450 required for 4-ABP N-hydroxylation and that another, yet to be identified, P450 is likely to be involved.

Based on these facts and the known association of chemical exposure with toxicities and cancer in humans, the safety of chemicals developed for use as drugs, food additives, herbicides and pesticides or that eventually end up as environmental pollutants is carefully assessed as to potential health or environmental hazards. Xenobiotic metabolizing enzymes are responsible for either mediating the toxicity of chemicals or protecting the organism by rapidly detoxifying chemicals to inert derivatives that can be eliminated. The most widely studied of these enzymes are the cytochromes P450 (P450s), which are responsible for oxidative metabolism.

P450s are usually involved in oxidation of foreign compounds, including therapeutically used drugs, and are the principal enzymes responsible for metabolic activation of carcinogens and toxins. P450s consist of a large superfamily of proteins (1) that includes, among others, four families, CYP1, CYP2, CYP3 and CYP4, that appear to be primarily involved in metabolism of xenobiotics. Studies of these enzymes are critical to understanding toxic mechanisms and in determining human risk assessment. However, there are marked species differences in the expression and catalytic activities of P450s that can have a significant impact when using rodent model systems in research and in testing of chemicals for safety in humans (2). In particular, P450s in the CYP2 family display considerable differences in expression, regulation and catalytic activities between humans and rodent animal models (3). This complicates interpretation of data and extrapolation of rodent studies to humans.

While there are a large number of P450s, only a rather limited group of enzymes primarily carry out metabolic activation of toxins and carcinogens. CYP1A1 and CYP1B1 activate polycyclic aromatic hydrocarbons and CYP2E1 metabolizes a large number of low molecular weight suspected cancer-causing agents. CYP1A2 carries out the N-hydroxylation of arylamine carcinogens and heterocyclic amine food mutagens (4,5). CYP1A2 is constitutively expressed in the liver of mice, rats and humans and is inducible by ligands of the aryl hydrocarbon receptor in all mammalian species analyzed. Interindividual differences in levels of expression of CYP1A2 have been found to be associated with susceptibility to colon cancer, especially when high consumption of food mutagens is considered (6). The association of CYP1A2 with carcinogenesis is largely assumed, based on in vitro metabolic studies with microsomes as well as with purified and cDNA-expressed P450s (7). However, it is still not certain whether CYP1A2 is required for carcinogenesis induced by arylamine carcinogens in the intact animal. CYP1A2-null mice constitute an appropriate in vivo model to address this issue (8,9). The validity of the CYP1A2-null mouse to study drug metabolism was established by demonstrating that CYP1A2 is required for rapid and efficient elimination of caffeine in vivo (9). CYP1A2 was previously shown by in vitro studies to be the principal low Km P450 responsible for the initial 3-demethylation step in caffeine catalysis (10,11). Thus, the CYP1A2-null mouse

Introduction

Most chemical carcinogens and toxins require metabolic activation by xenobiotic metabolizing enzymes in order to exert their harmful effects. This usually involves metabolism to an unstable electrophoric derivative that can react with nucleophiles in macromolecules, including DNA. Carcinogen–DNA adducts can form that can result in mutations in oncogenes and tumor suppressor genes leading to cell transformation.

Abbreviations: 4-ABP, 4-aminobiphenyl; DMSO, dimethylsulfoxide; P450s, cytochromes P450; PhIP, 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine.
is an appropriate investigatory model for assessing whether CYP1A2 is required for carcinogenesis in vivo using the standard rodent carcinogen bioassays.

The arylamine 4-aminobiphenyl (4-ABP), a human carcinogen, is found in the environment and is a major component of tobacco smoke. Evidence of human exposure is found both in hemoglobin adducts (12) and in DNA adducts in the bladder (13) and lung (14,15). Metabolism of 4-ABP by N-acetylation and C- and N-hydroxylation occurs primarily in the liver in rodents (16). Metabolic activation of the N-hydroxy metabolite is mediated by esterification by sulfotransferase or N-acetyltransferase. Adult and neonatal mice are also known to express phase II activating enzymes such as acetyltransferase and sulfotransferase at levels comparable with that of humans and
rats (17,18). In the present study, the role of CYP1A2 in 4-ABP carcinogenesis was assessed using both CYP1A2-null and wild-type mice in the neonatal carcinogen bioassay (18). The neonatal bioassay is known to yield liver tumors in mice using 4-ABP.

### Materials and methods

**Chemicals**

4-ABP was purchased from Aldrich Chemical Co. (Milwaukee, WI), repurified with silica gel chromatography using benzene:ethyl acetate (9:1) and then recrystallized from hot hexane. The purity was determined by silica gel chromatography using benzene:ethyl acetate (9:1) and then HPLC. 4-ABP was purchased from Aldrich Chemical Co. (Milwaukee, WI), repurified with 4-ABP.

**Animals**

Characterization of CYP1A2-null (9) and AHR-null (19) mice has been done. They are derived from a mixed background of 129/Sv and C57BL/6 mouse strains. Animals were maintained in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, 1996) under an animal study proposal approved by the NCI Animal Care and Use Committee.

**Animal treatment**

4-ABP was dissolved in dimethylsulfoxide (DMSO) at 20 and 40 mM, which we refer to as the low and high dose groups, respectively. Mice were injected i.p. with 10 and 20 l of 4-ABP solution on 8 and 15 days of age, respectively. Mice were injected with 10 and 20 l of 4-ABP solution on 8 and 15 days of age, respectively.

**Pathology**

All mice were necropsied and body and liver weights recorded. Two representative sections were obtained from the left and median liver lobes from each mouse. Grossly visible liver tumors that were not in the same plane as these representative sections were also sectioned. Proliferative liver lesions (hepatocellular foci, adenomas and carcinomas) were diagnosed according to standard and current criteria (20,21) and the number and types of foci were determined per unit area of the liver section (no./cm²). Statistics were carried out using Student’s t-test.

**Microsomes**

Liver microsomes were prepared from CYP1A2-null, AHR-null and wild-type mice that had been treated with 4-ABP or DMSO as a control on 8 and 15 days of age, using the same regimen as the high dose 4-ABP neonatal bioassay, and killed on day 16. The AHR-null mice were used as a control for no expression of CYP1A1 in both metabolite assays and western blotting analyses (see below). Protein concentration was determined by use of the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL) using bovine serum albumin as standard.

**Liver weight**

The final mean body weights were not significantly different regardless of sex, genotype and treatment among mice killed at 16 months of age (Table I). Liver weights were also not significantly different, except in the case of females treated with low dose 4-ABP as compared with the respective wild-type group.

**Proliferative liver lesions**

Incidences of liver lesions revealed that 4-ABP induced hepatocellular preneoplastic foci (Figure 1A–C), adenomas and carcinomas (Figure 1D) and that the incidences were higher in those animals treated with 4-ABP than in the untreated control groups (Table II). The incidences were higher in male mice than in female mice. Interestingly, no differences in the incidences of hepatocellular foci and adenomas by genotype or 4-ABP dose were found except in male mice treated with low dose 4-ABP. In this case, significantly lower incidences were obtained as compared with their respective wild-type groups. Hepatocellular carcinomas were also observed in

### Table I. Liver weights in Cyp1a2 null and +/+ mice injected with 4-ABP perinatally and killed at 16 months

<table>
<thead>
<tr>
<th>Sex</th>
<th>Group</th>
<th>No. of mice</th>
<th>Final body wt (g)</th>
<th>Liver wt Absolute (g)</th>
<th>Relative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>ABP low –/–</td>
<td>27</td>
<td>39.8 ± 8.0*</td>
<td>2.7 ± 1.4</td>
<td>6.7 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>ABP low +/+</td>
<td>30</td>
<td>38.3 ± 7.5</td>
<td>2.8 ± 2.0</td>
<td>7.5 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>ABP high –/–</td>
<td>42</td>
<td>43.6 ± 10.4</td>
<td>2.8 ± 1.5</td>
<td>6.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>ABP high +/+</td>
<td>26</td>
<td>41.9 ± 5.7</td>
<td>2.7 ± 1.2</td>
<td>6.8 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>DMSO⁶</td>
<td>12</td>
<td>48.9 ± 5.9</td>
<td>2.4 ± 0.8</td>
<td>4.9 ± 1.5</td>
</tr>
<tr>
<td>Female</td>
<td>ABP low –/–</td>
<td>25</td>
<td>35.4 ± 11.9</td>
<td>1.8 ± 0.3β</td>
<td>5.3 ± 1.1⁵</td>
</tr>
<tr>
<td></td>
<td>ABP low +/+</td>
<td>23</td>
<td>37.4 ± 9.4</td>
<td>1.5 ± 0.5</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>ABP high –/–</td>
<td>27</td>
<td>43.8 ± 9.5</td>
<td>1.7 ± 0.5</td>
<td>4.0 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>ABP high +/+</td>
<td>33</td>
<td>40.4 ± 10.0</td>
<td>1.6 ± 0.4</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>DMSO⁶</td>
<td>25</td>
<td>41.5 ± 9.7</td>
<td>1.5 ± 0.3</td>
<td>3.8 ± 0.8</td>
</tr>
</tbody>
</table>

*Data are means ± SD.

†Includes DMSO-injected –/–, +/+ and +/+ mice.

§Significantly different from their respective wild-type groups at P < 0.01.

### Table II. Incidences and quantitative data of proliferative liver lesions in Cyp1a2 null and +/+ mice injected with 4-ABP perinatally and killed at 16 months

<table>
<thead>
<tr>
<th>Sex</th>
<th>Group</th>
<th>No. of mice</th>
<th>Incidences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Foci</td>
</tr>
<tr>
<td>Male</td>
<td>ABP low –/–</td>
<td>27</td>
<td>13 (48)²</td>
</tr>
<tr>
<td></td>
<td>ABP low +/+</td>
<td>30</td>
<td>21 (70)³</td>
</tr>
<tr>
<td></td>
<td>ABP high –/–</td>
<td>42</td>
<td>29 (69)⁴</td>
</tr>
<tr>
<td>Female</td>
<td>ABP low –/–</td>
<td>25</td>
<td>18 (69)⁴</td>
</tr>
<tr>
<td></td>
<td>ABP low +/+</td>
<td>23</td>
<td>2 (17)²</td>
</tr>
<tr>
<td></td>
<td>ABP high –/–</td>
<td>27</td>
<td>14 (52)</td>
</tr>
<tr>
<td></td>
<td>ABP high +/+</td>
<td>33</td>
<td>12 (36)</td>
</tr>
<tr>
<td></td>
<td>DMSO⁶</td>
<td>25</td>
<td>7 (28)</td>
</tr>
</tbody>
</table>

³Hepatocellular carcinoma.

⁴Includes DMSO-injected –/–, +/+ and +/+ mice.

⁵Numbers in parentheses show the percentage incidence.

⁶Significantly different from their respective wild-type groups at P < 0.05.

⁷Significantly different from the respective DMSO groups at P < 0.05 and 0.01.

### 4-ABP metabolite assays

Microsomal N-hydroxylation of 4-ABP was measured in an incubation mixture containing 100 mM potassium phosphate buffer (pH 7.5), 0.05 mM EDTA, 5 mM glucose 6-phosphate, 0.4 mM NADP⁺, 0.3 mM NAD⁺, 1 U/ml glucose 6-phosphate dehydrogenase, 0.1 mM [³H]4-ABP and 1 mg liver microsomal protein/ml. The mixture was preincubated at 37°C for 5 min before addition of substrate. The incubation was continued for 10 min, terminated by the addition of an equal volume of cold methanol and then analyzed by HPLC (22).

**Western blotting**

Liver microsomal proteins (13 μg) obtained from 16-day-old CYP1A2-null, AHR-null and wild-type pups were run on 8% SDS–polyacrylamide gels and proteins were transferred to nitrocellulose membranes for western blotting (Schleicher & Schuell, Keene, NH). The blots were developed using rabbit anti-rat CYP1A1, CYP2B1 and CYP2C11 (Gentest Corp., Woburn, MA) or anti-mouse CYP1B1 antibodies (kindly provided by Dr Colin R.Jefcoate, Madison, WI) and secondary antibody coupled with horseradish peroxidase for enhanced chemiluminescence detection (ECL; Amersham, Arlington Heights, IL). The anti-rat CYP1A1 antibody cross-reacts with mouse CYP1A1 and CYP1A2. The anti-rat 2B1 and 2C11 antibodies cross-react with mouse P450s belonging to the CYP2B and 2C families, respectively.
male mice, but no differences in incidence were seen when comparing either genotype or 4-ABP dose used.

**Liver foci**

Although overall differences were not significant in the incidences of all liver foci (Tables II and III), significant differences in multiplicity were found for specific types of foci when CYP1A2-null and their respective wild-type mice were compared (Table III). For instance, eosinophilic foci (Figure 1B) were found at significantly higher incidences in CYP1A2-null mice, particularly in males treated with low dose 4-ABP and females treated with the high dose. Lower incidences of basophilic foci (Figure 1A) were seen in null male mice treated with both low and high 4-ABP doses. The CYP1A2-null mice treated with the high dose of 4-ABP appeared to have more clear cell foci and this is especially evident in male mice. Foci with intracytoplasmic eosinophilic inclusions (Figure 1C) were seen at lower incidences in both male and female mice lacking CYP1A2 expression, regardless of 4-ABP dose. These inclusions have been described in mouse foci and adenomas (20).

**Stomach**

Gastric fundic plaques (Figure 1E) were found in 44–50% of the male and female CYP1A2-null mice but in only 0–12% of the wild-type mice, without effect of 4-ABP exposure, as previously reported (23). These lesions often contained dense eosinophilic cytoplasm and intracytoplasmic inclusions of various sizes (Figure 1F).

**4-ABP metabolite study**

Arylamine N-hydroxylation activity was measured using liver microsomes prepared from CYP1A2-null and wild-type mice that had been treated with either DMSO as a control or with high dose 4-ABP. AHR-null homozygous mice were also similarly treated and the microsomes used for the activity assay (Table IV). The most interesting finding in these metabolite assays is that both CYP1A2-null and AHR-null mouse liver microsomes exhibited about half the activity of the wild-type liver microsomes. These results clearly demonstrate that CYP1A2 is not the primary P450 metabolizing 4-ABP in these mice, since both CYP1A2-null and AHR-null mice lack expression of CYP1A2 (see below). Further, CYP1A1 is not the P450 metabolizing 4-ABP, since AHR-null mice, which lack CYP1A1 expression, exhibited microsomal arylamine N-hydroxylation activity similar to those of CYP1A2-null mice. The lack of CYP1A1 expression in AHR-null mice was confirmed by western blotting using liver microsomes (see below). All three mouse lines (wild-type, CYP1A2-null and AHR-null) had 1.2- to 1.4-fold higher 4-ABP metabolic activity (CYP1A1) were seen in null male mice treated with both low and high 4-ABP doses. Lower incidences of basophilic foci (Figure 1A) were seen in null male mice treated with both low and high 4-ABP doses. The CYP1A2-null mice treated with the high dose of 4-ABP appeared to have more clear cell foci and this is especially evident in male mice. Foci with intracytoplasmic eosinophilic inclusions (Figure 1C) were seen at lower incidences in both male and female mice lacking CYP1A2 expression, regardless of 4-ABP dose. These inclusions have been described in mouse foci and adenomas (20).

**Table IV. Arylamine N-hydroxylation in liver microsomes from Cyp1a2 −/− and +/+ mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DMSO</th>
<th>ABP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp1a2 +/+</td>
<td>172 ± 7</td>
<td>211 ± 18</td>
</tr>
<tr>
<td>Cyp1a2 −/−</td>
<td>96 ± 4</td>
<td>118 ± 16</td>
</tr>
<tr>
<td>Ahr −/−</td>
<td>79 ± 5</td>
<td>113 ± 2</td>
</tr>
</tbody>
</table>

*Data are means ± SD.

**Fig. 2.** Western immunoblot analysis of CYP1A1 and CYP1A2 in wild-type (+/+), CYP1A2-null (1A2−/−) and AHR-null (AHR−/−) mice administered 4-ABP (ABP) and DMSO vehicle. Recombinant mouse CYP1A1 and CYP1A2 were used as positive controls.
intensity regardless of treatment or genotype (data not shown). Further, CYP1B1 expression was not detected in any of the microsomes examined (data not shown).

Discussion

4-ABP has been shown to be metabolically activated primarily by CYP1A2 in rat and human liver (22). In this paper, the neonatal mouse bioassay was carried out in order to assess the susceptibility of the CYP1A2-null mouse to 4-ABP and to evaluate if CYP1A2 is also the primary metabolic activator of 4-ABP in mouse liver. Treatment with 4-ABP induced liver foci and hepatocellular adenoma of the liver in both the low and high dose groups. However, no differences in incidence were found between genotypes (wild-type and CYP1A2-null mouse), irrespective of the dose. Further, hepatocellular carcinomas observed in male mice showed no differences in incidence between genotypes. These results suggest that a CYP1A2-independent pathway of metabolism of 4-ABP may be involved in hepatocarcinogenesis induced by this carcinogen. In fact, the 4-ABP metabolite assay results clearly demonstrate that CYP1A2 is not the only P450 metabolizing this chemical in mice; approximately half of the activity is due to a CYP1A2-independent pathway. The liver DNA adduct levels did not reveal any differences between CYP1A2-null and wild-type mice (unpublished data), which also supports the involvement of a CYP1A2-independent pathway(s) in 4-ABP metabolism. Because no dose dependency was obtained in this hepatocarcinogenicity study, the CYP1A2-independent 4-ABP metabolic pathway may be more easily activated by a dose lower than the 600 nmol used in this study. The incidence of liver foci and hepatocellular adenoma was higher in male than female mice and hepatocellular carcinomas were observed mostly in male mice. These results may be another good example of male mice being more susceptible to hepatocarcinogenesis than female mice. The tumor-promoting effect of testosterone has been well documented in mice (24,25). Alternatively, these results suggest that the CYP1A2-independent 4-ABP metabolic pathway may be hormonally influenced.

Although no overall differences were found in liver foci incidences between genotypes, significant differences in multiplicity for specific types of foci were found. For example, eosinophilic and clear cell foci were found at higher levels and foci with inclusions were observed at lower incidences in both male and female mice lacking CYP1A2 expression. These results imply that the CYP1A2-dependent 4-ABP metabolic pathway may contribute to production of some types of liver foci in either a positive or negative fashion. Actually, the 4-ABP metabolite data using wild-type mice showed that about half the activity may be due to CYP1A2. Alternatively, it remains a possibility that induction of specific foci by the CYP1A2-independent pathway may be modified by CYP1A2.

Western blotting results clearly demonstrated that there is no CYP1A2 expression in either CYP1A2-null or AHR-null mice nor is there induction of CYP1A1 by 4-ABP. This is further confirmed by the results of the hepatocarcinogenesis study showing that 4-ABP appears to be metabolically activated by CYP1A2 and a yet to be identified P450 in mice. Metabolism studies suggest that the unknown P450 responsible for metabolism of 4-ABP may be induced by treatment with the substrate. Western blotting using anti-rat 2B1 and 2C11 antibodies, however, did not reveal any differences in levels of cross-reacting P450s on 4-ABP treatment in livers of mice from the three genotypes. CYP1A2 levels were also not induced by 4-ABP, as seen in wild-type mice. These results suggest that the second P450 responsible for 4-ABP metabolism in mouse is not a member of the CYP2B or CYP2C families. Alternatively, the P450 may be a member of one of these families, but does not cross-react with the anti-rat P450 antibodies used. This would appear unlikely, since rat and mice P450s exhibit ~85–95% amino acid sequence similarity within families. It also remains a possibility that levels of induction (1.2- to 1.4-fold increase relative to wild-type as based on the metabolism studies) are not large enough so that it is masked by cross-reacting P450s of the same mobility using the standard western blotting technique. It remains to be determined which P450, in addition to CYP1A2, is capable of metabolically activating 4-ABP in the mouse.

The metabolism of 4-ABP has been studied using rats and humans and is known to be mainly carried out by CYP1A2. In rats, CYP1A2 is induced by treatment with 4-ABP. Our current study clearly suggests that there are species differences in 4-ABP metabolic activation among rats, humans and mice. In mice, CYP1A2 does not seem to be induced by 4-ABP and is not the primary enzyme responsible for metabolizing 4-ABP. However, CYP1A2 is expressed in neonatal mouse liver, as seen in the western blotting results, as are phase II activating enzymes such as acetyltransferase and sulfotransferase that metabolically activate the 4-ABP N-hydroxy metabolite (17,18). Thus, the metabolic machinery required for 4-ABP activation is present in the neonate. However, these results obtained using neonatal mice might not be directly comparable with humans, in which biomarker studies associated with 4-ABP exposure were mainly carried out in adults (13–15). Clear species differences have been reported in the metabolism of 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP), a protein-derived pyrolysate component present in cooked foods (26,27). In rats, PhIP induces CYP1A1, CYP1A2 and an unidentified P450 enzyme with a molecular weight of ~51 kDa in the liver and CYP1A1 in the kidney, whereas none of these are induced by PhIP in mice, hamsters and guinea pigs (27). Although the unidentified P450 is not induced in mice by PhIP treatment, there is a possibility that this P450 may be identical to the one responsible for 4-ABP metabolism in the current study.

In conclusion, our current studies suggest that carcinogenesis by arylamines in mice is dependent on more than one P450, even though CYP1A2 has been shown by in vitro studies to be the main P450 responsible for 4-ABP activation in rats and humans (22). The carcinogen bioassay would suggest that CYP1A2 is not the sole P450 required for 4-ABP carcinogenesis in the mouse when the neonatal bioassay is used. These studies clearly demonstrate that the route of administration and species difference in metabolism could hamper our ability to extrapolate mouse carcinogenesis data to humans.

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References


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