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Decrease in 4-Aminobiphenyl-Induced Methemoglobinemia in *Cyp1a2(−/−)* Knockout Mice[1]

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Methemoglobin formation, as well as hemoglobin or DNA adducts, are useful biomarkers of occupational exposure to certain arylamines. It has been suggested that, in liver from animals not treated with a cytochrome P450 (CYP) inducer, hepatic CYP1A2 is the major P450 involved in N-hydroxylation. This is the first step in the metabolic activation of many arylamines, such as the human urinary bladder carcinogen 4-aminobiphenyl (ABP). The product of this catalytic step, *N*-hydroxy-4-ABP, reacts in the blood with oxyhemoglobin to form methemoglobin and nitrosobiphenyl. We therefore examined the role of CYP1A2 in causing methemoglobinemia in ABP-treated *Cyp1a2(−/−)* knockout mice. Application of ABP (100 μmol/kg body wt) to the skin resulted in a marked depletion in the levels of the hepatic thiols (reduced glutathione and cysteine) after 2 h, which rebounded to basal levels 24 h later, and we found no differences between the *Cyp1a2(−/−)* and wild-type *Cyp1a2(+/+)* animals. Unexpectedly, the methemoglobin levels were significantly ($p < 0.05$) higher in *Cyp1a2(−/−)* than *Cyp1a2(+/+)* mice at 2, 7, and 24 h following topical ABP. Treatment with dioxin, 24 h prior to ABP, decreased methemoglobin levels by about half at each of the time points in both the *Cyp1a2(−/−)* and *Cyp1a2(+/+)* mice. These data suggest that CYP1A2 does not play a positive role in methemoglobin formation via the activation of ABP; rather, the absence of CYP1A2 enhances ABP-induced methemoglobinemia. Because liver CYP1A2 levels are known to vary more than 60-fold between humans, our findings may be relevant to patients who are exposed to arylamines in the workplace. © 2002 Elsevier Science (USA)

Key Words: **methemoglobinemia; 4-aminobiphenyl; biomarkers of exposure; CYP1A2; dioxin; hepatic cysteine levels; reduced glutathione.**

Exposures to certain environmental *N*-heterocyclic and aromatic amine compounds pose a threat to human health. The most common route for occupational human exposure to aromatic amines such as 4-aminobiphenyl (ABP[3]) is transdermal (Scott, 1962; NIOSH, 1990). ABP and other aromatic amines are efficiently absorbed through the skin and have been given a "skin notation" by the American Conference of Governmental Industrial Hygienists. Workers exposed to these materials have been documented to have a tremendously excessive risk of urinary bladder cancer; observed relative risks range from 10-fold to approximately 90-fold, depending on the study (Vineis *et al.*, 1994). In addition, aromatic amines such as ABP, 2-naphthylamine, and *o*-toluidine are known to be components of tobacco smoke. It has been documented (Talaska *et al.*, 1991, 1993) that *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl is the major DNA adduct in the urothelium of tobacco smokers. In mouse studies, topical ABP has been shown to produce ABP–DNA adducts in lung, liver, skin, and urinary bladder (Underwood *et al.*, 1997).

The major biotransformation pathways for ABP are illustrated in Fig. 1. The metabolic balance between the detoxification and activation pathways for ABP are believed to modulate its toxicity. ABP detoxification occurs primarily in the liver, with major pathways thought to involve *N*-acetyltransferase-2 and cytochrome P450 (CYP)-mediated ring hydroxylation (Turesky *et al.*, 1998; Probst-Hensch *et al.*, 2000). N-hydroxylation is the primary initial step in the activation pathway, catalyzed by one or more forms of CYP. The *N*-hydroxy metabolite may be detoxified by N-acetylation or undergo further activation by O-acetylation, O-sulfation, or O-glucuronidation. The former two are considered to be highly unstable species that can react with protein and DNA under physiological conditions. All three of the *O*-conjugates are unstable under acidic conditions, such as may occur in the urinary bladder. These compounds may undergo acid-mediated decomposition to form the electrophilic nitrenium cation, which is able to form adducts with DNA, hemoglobin, and protein or nonprotein sulfhydryls. (Kadlubar and Badawi, 1995; Guengerich *et al.*, 1995; Landi *et al.*, 1996). Alternatively, the aryl hydroxylamine may enter the blood and un-

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[3] Abbreviations used: ABP, 4-aminobiphenyl; FMO, flavin-containing monooxygenase; OTC, ornithine carbamoyltransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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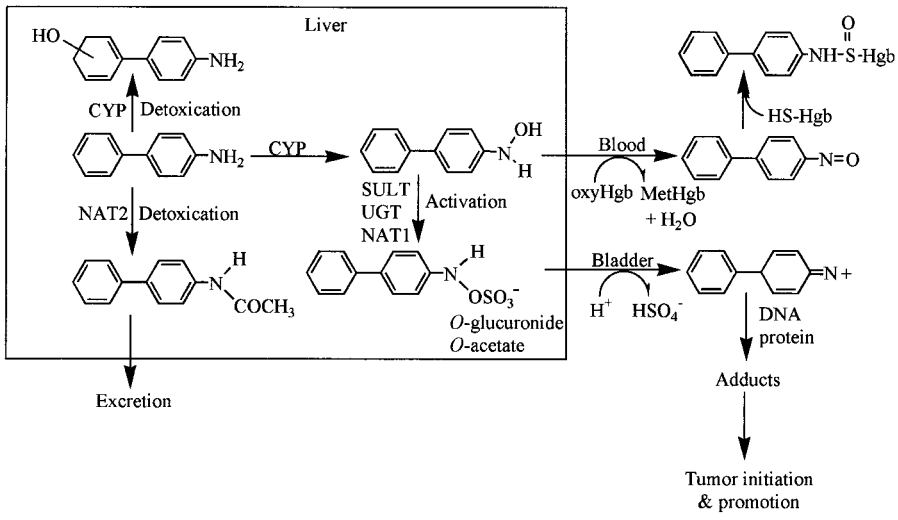

FIG. 1. Major pathways of hepatic ABP metabolism and further reactions of ABP metabolites in blood and urinary bladder. CYP, presumably a cytochrome P450 reaction [although FMO (flavin monooxygenase) is also possible]; NAT2, *N*-acetyltransferase-2; NAT1, *N*-acetyltransferase-1; SULT, sulfotransferase; UGT, UDP glucuronosyltransferase; Hgb, hemoglobin; metHgb, methemoglobin; oxyHgb, oxyhemoglobin.

dergo iron-catalyzed cooxidation with oxyhemoglobin to produce methemoglobin and *N*-nitrosobiphenyl. The latter has been shown to react with cysteinyl sulfhydryl moieties of hemoglobin to produce ABP–hemoglobin adducts in humans (Green *et al.*, 1984) (Fig. 1).

CYP1A1, CYP1A2, and CYP1B1 are generally believed to be required for the mutagenic activation and, presumably, the carcinogenic activation of these compounds (Turesky *et al.*, 1998); in the liver of animals not treated with a CYP inducer, CYP1A1 and CYP1B1 levels are extremely low, such that CYP1A2 is the major activating enzyme (Eaton *et al.*, 1995; Nebert *et al.*, 1996). Tobacco smoke-derived *N*-heterocyclic compounds (e.g., dibenz[*c,g*]carbazole) and *N*-nitrosamines [e.g., 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)], are activated to mutagenic and carcinogenic products by CYP1A2 *in vitro* (Crespi *et al.*, 1991; Smith *et al.*, 1996). It has been suggested that several human occupational urinary bladder carcinogens (e.g., ABP, 2-naphthylamine, and methylene-*bis*-2-chloroaniline) are also metabolically activated by CYP1A2 (Butler *et al.*, 1989a,b; Eaton *et al.*, 1995). ABP has been of special concern regarding human safety, due to its presence in the environment, the workplace, and tobacco smoke.

In human populations, differences in hepatic CYP1A2 protein levels and enzyme activities vary more than 60-fold (Eaton *et al.*, 1995; Nebert *et al.*, 1996). The *CYP1A2* gene is highly polymorphic (Oscarson *et al.*, 2001), although no DNA sequence variants so far discovered can explain these large interindividual differences in protein and activity levels. Because CYP1A2 is believed to be important in the metabolic activation of arylamines, these genetic differences would suggest that individuals with the highest constitutive CYP1A2 might be at greatest risk to toxicity and cancer, if chronically exposed to these environmental agents. In this regard, certain forms of cancer are associated with ingestion of food-derived hetercyclic amines–such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,4,8-trimethylimidazo-[4,5-*f*]quinoxaline (DiMeIQx), and 2-amino-3-dimethylimidazo[4,5-*f*]quinoline (IQ)–produced at high temperatures in the cooking of meat and fish (Felton *et al.*, 1997).

To begin to understand the importance of CYP1A2 in the activation of aromatic amines, we have ablated its function by gene targeting. Cyp1a2(−/−) mice do not synthesize CYP1A2 protein and have previously been shown to have defects in drug metabolism and caffeine clearance and to be completely resistant to experimentally induced uroporphyria (Liang *et al.*, 1996; Buters *et al.*, 1996; Sinclair *et al.*, 1998, 2000). In order to examine the role of CYP1A2 in the metabolic activation of ABP, we compared levels of hepatic reduced glutathione (GSH) and cysteine, as well as methemoglobinemia in *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice. These parameters are indicative of the hepatic production of the ABP metabolite *N*-hydroxy-4-ABP and of its potential for transport to the bloodstream.

METHODS

Chemicals

TCDD was purchased from Accustandard (New Haven, CT). All other chemicals and reagents were obtained from either Aldrich Chemical Company (Milwaukee, WI) or Sigma Chemical Company (St. Louis, MO) as the highest available grades. ABP was a kind gift from Dr. Fred Kadlubar (National Center for Toxicology Research, Jefferson, AR).

Mice

All animal experiments were conducted in accordance with the National Institutes of Health standards for the care and use of experimental animals and

the University of Cincinnati Medical Center (UCMC) Institutional Animal Care and Use Committee (IACUC). Generation of the *Cyp1a2(−/−)* mouse line, starting from the C57BL/6J and 129/J inbred strains, has been described (Liang *et al.*, 1996), and the *Cyp1a2(−/−)* colony that we maintain has been backcrossed into C57BL/6J for eight generations; this ensures that the knockout genotype resides in a genetic background that is >99.8% C57BL/6J (Nebert *et al.*, 2000). If the mouse genome contains 30,000 genes, this value would represent ~29,940 C57BL/6J genes and ~60 129/J genes (these percentages are purely mathematical calculations, and the actual amount of genetically homogeneous background may become asymptotic as the mouse lines approach 98%). Accordingly, we are justified in using, as the *Cyp1a2(+/+)* wild-type control, age-matched C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME).

ABP (100 μmol/kg body wt) was applied topically (in 25 μl acetone) to the shaved dorsal skin of male mice weighing about 25 g. Some of the mice were treated intraperitoneally with TCDD (15 μg/kg body wt) in corn oil 24 h prior to ABP administration. Control mice received the vehicle alone. This dosage of ABP is low relative to dosages often used to produce tumors.

Hepatic Thiols and Toxicity

Mice were killed by carbon dioxide asphyxiation followed by cervical dislocation, liver tissue was excised and processed, and hepatic GSH was determined by the fluorometric procedure of Senft *et al.* (2000). Hepatic cysteine was assayed and quantified by an HPLC procedure with fluorescence detection (Shertzer *et al.*, 1995). For ornithine carbamoyltransferase (OTC) activity, blood was collected by cardiac puncture in heparinized syringes, centrifuged, and the serum was assayed for OTC activity (Vassef, 1978).

Methemoglobin

Blood was obtained from the tail vein at 2, 7, and 24 h following ABP treatment. A 10-μl aliquot of blood was collected in a heparinized capillary tube and diluted into 0.99 ml of 50 mM potassium phosphate buffer containing 5 mM DPTA and 0.05% Triton X-100 (pH 7.0). After hemolysis, the absorbance spectrum of the blood was scanned between 550 and 720 nm, and absorbance values were determined at 577, 630, and 700 nm. Total hemoglobin and methemoglobin levels were determined by the method of Winterbourn (1985).

Statistics

All assays were performed in duplicate or triplicate, and the average values were considered as one independent determination. Statistical differences between group mean values were determined by a one-way ANOVA, followed by a Student–Newman–Keuls test for a pairwise comparison of means. Statistics were performed using SigmaStat Statistical Analysis software (SPSS Inc., Chicago, IL).

Biohazard Precaution

TCDD and ABP are both highly toxic and likely human carcinogens. All personnel were instructed as to safe handling procedures. Lab coats, gloves, and masks were worn at all times, and contaminated materials were collected separately for disposal by the Hazardous Waste Unit or by independent contractors. TCDD-treated mice were housed separately, and their carcasses were treated as contaminated biological materials.

RESULTS

Hepatic Thiols and Toxicity

Topical application of ABP produced a 60 to 80% depletion of the hepatic thiols (GSH and cysteine) at 2 h, followed by recovery to basal levels by 24 h (Fig. 2). Surprisingly, we found no significant differences in the depletion of hepatic thiols between the *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice, suggesting that CYP1A2 might not be critical for this process. Consistent with these findings were the serum enzyme activities of OTC, a specific indicator of hepatocellular damage (Fig. 3). We found no differences in serum OTC activities following ABP administration, between *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice, or even following pretreatment with dioxin, a potent inducer for CYP1A1 and CYP1A2.

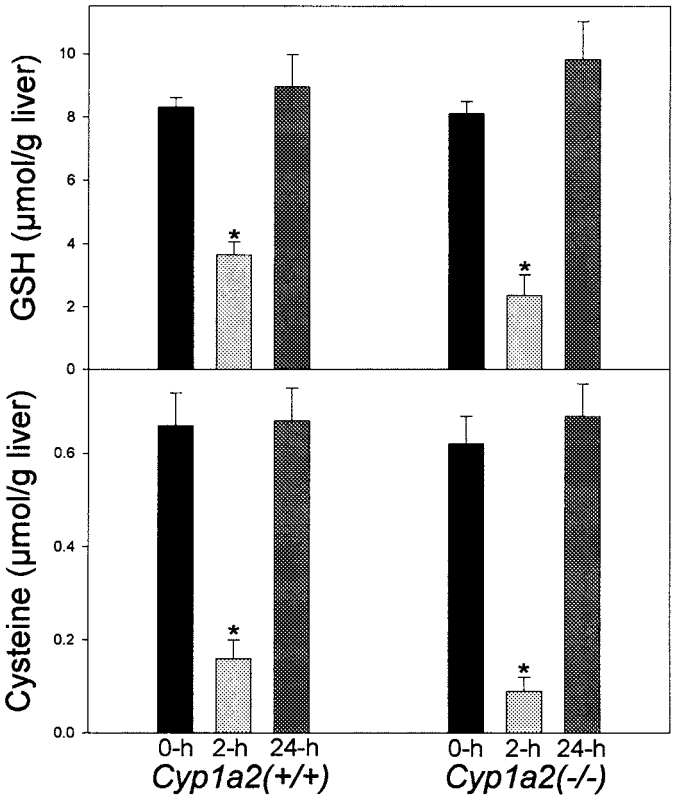

FIG. 2. Hepatic thiol levels following treatment with ABP. *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice were treated topically with the acetone vehicle alone (control, 0 h) or ABP (100 μmol/kg) for 2 or 24 h before liver measurements. Values are expressed as means ± SEM ($N = 8$). *Significantly different means from those obtained in control mice ($p < 0.05$).

Extrahepatic Targets

We next examined the potential for ABP metabolites to react with extrahepatic targets. First, GSH concentrations in the red cell were measured, in order to determine if a hepatic thiol-reacting intermediate had been released to the blood. Such an intermediate could be 4-nitrosobiphenyl or an arene oxide (Fig. 1). We found that GSH levels remained constant at 0.11 ± 0.01 nmol GSH/nmol total hemoglobin in *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice under all treatment conditions (controls, 2, 7, and 24 h after ABP, with or without TCDD). We conclude that dioxin-inducible or constitutive CYP1A2 has no detectable effect on red cell GSH in ABP-treated mice.

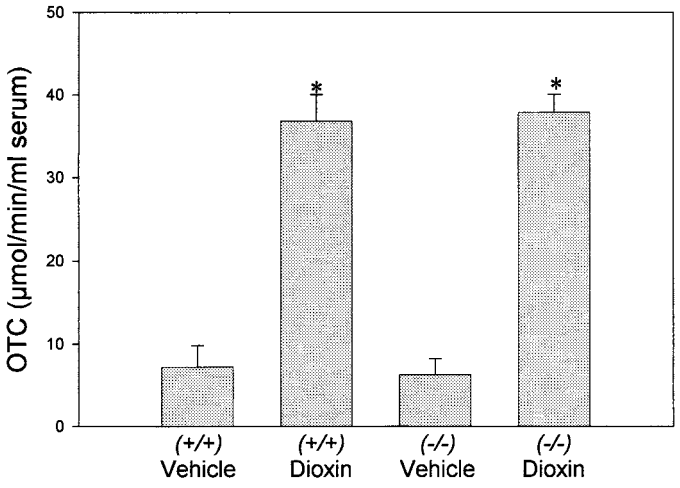

FIG. 3. Serum OTC activities following treatment with topical ABP. Mice were given intraperitoneally either dioxin (15 μg/kg) in 25 μl corn oil, or vehicle alone. After 24 h, mice were treated with ABP (100 μmol/kg), and the mice were killed 24 h later. The results are expressed as mean values ± SEM ($N = 8$). *Significantly different means from those in vehicle-treated mice ($p < 0.05$).

Next, we examined methemoglobin formation–believed to represent the capacity of *N*-hydroxy-4-ABP to cooxidize with oxyhemoglobin to form methemoglobin and 4-nitrosobiphenyl. As shown in Fig. 4, ABP at 2 h produced a rapid increase in methemoglobin levels in *Cyp1a2(+/+)* mice. By 7 h, the amount of methemoglobin had returned to near baseline levels, but remained slightly elevated even at 24 h. Following ABP treatment of *Cyp1a2(−/−)* mice, with or without dioxin pretreatment, methemoglobin levels at 2 h were significantly higher than those observed in *Cyp1a2(+/+)* mice and returned toward baseline levels more slowly. These surprising results suggest that either the presence of CYP1A2 decreases methemoglobin formation or that the absence of CYP1A2 actually enhances ABP-induced methemoglobinemia.

Pretreatment with dioxin diminished ABP-elicited methemoglobin levels to about half those observed in either *Cyp1a2(+/+)* or *Cyp1a2(−/−)* mice not receiving dioxin. These data indicate that some of the ABP detoxification pathway is inducible by dioxin. It is likely in this regard that ring hydroxylation by CYP1B1 (Gonzalez, 2001; Murray *et al.*, 2001) or N-glucuronidation by UGT1A6 or UGT1A7 (Mackenzie *et al.*, 1997; Ritter, 2000)–all dioxin-inducible enzymes–may be involved.

DISCUSSION

The aromatic amine ABP is a recognized carcinogen in rodents, canines, and humans. The primary target organs for ABP tumorigenesis include liver in rats, liver and urinary bladder in mice, and urinary bladder in dogs and humans. Whereas the number of persons working in occupations involving exposure to aromatic amines is small, they are at extreme risk for urinary bladder cancer. Smokers are also exposed to aromatic amines and have a three- to sevenfold increased risk of urinary bladder cancer and three- to sevenfold higher levels of ABP–DNA adducts in the urinary bladder (Talaska *et al.*, 1991; Hsu *et al.*, 1997).

Specific ABP–hemoglobin adducts have been detected (Green *et al.*, 1984; Bryant *et al.*, 1987). These adducts are significantly higher in smokers than in nonsmokers and are higher in patients with the slow-acetylator phenotype than with the rapid-acetylator phenotype (Vineis *et al.*, 1994); these data are consistent with the NAT2 detoxification pathway illustrated in Fig. 1. Reliable biomarker data about human exposure to ABP are derived from several sources. In their review, Skipper and Tannenbaum (1994) noted that ABP–hemoglobin adducts are detectable in persons without any documented exposure, suggesting a ubiquitous environmental presence.

From numerous *in vitro* studies using microsomes and purified cytochrome P450 monooxygenases from human and rat liver, it had been concluded that cytochromes P450 in the CYP1 family, primarily CYP1A2, are the major P450s responsible for the N-hydroxylation of ABP (Butler *et al.*, 1989a,b Hammons *et al.*, 1991; Landi *et al.*, 1996). It is beginning to appear, however, that the relative importance of CYP1A2 in this pathway may have been overestimated. For example, Kimura *et al.* (1999) recently found that *Cyp1a2(+/+)* and *Cyp1a2(−/−)* mice do not show a significant difference in ABP-induced hepatocellular adenoma, carcinoma, or preneoplasic foci; these authors suggest that, while CYP1A2 may be primarily responsible for the initial N-hydroxylation of ABP required for metabolic activation, CYP1A2 might play an additional role in modulating the toxicity and carcinogenicity

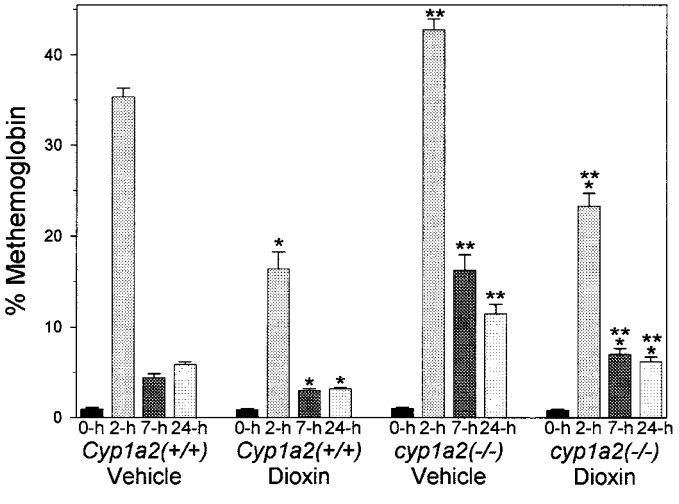

FIG. 4. Methemoglobin formation following treatment with ABP. The same treatment regimens described in the legends to Figs. 2 and 3 were carried out. Results are expressed as mean values ± SEM ($N = 8$). *Significantly different from the mean values in corn oil-treated mice ($p < 0.05$). **Significantly different from the mean values in *Cyp1a2(+/+)* mice ($p < 0.05$).

after the initial activation. The data in the present report, however, indicate even further that CYP1A2 is not the primary P450 involved in the N-hydroxylation of ABP. Moreover, we would suggest that other cytochromes P450 or a flavin-containing monooxygenase (FMO) might be involved in the metabolic activation of ABP. Further evidence comes indirectly from the report of Underwood *et al.* (1997), in which ABP–DNA adducts were found in mouse skin, an organ that does not contain CYP1A2. Our findings in the present study would also support a role for cytochromes P450 other than CYP1A2, or for an FMO, in the metabolic activation of ABP that leads to both hepatic thiol depletion and methemoglobin formation.

From a theoretical perspective, the results for ABP-induced methemoglobinemia could be explained by a CYP1A2-mediated pathway for ABP that leads away from the production of the aryl hydroxylamine intermediate. The only other likely metabolic pathway (Fig. 1) would be a ring oxidation, ultimately resulting in phenols or epoxides, followed by formation of a dihydroxy (*trans*-diol) metabolite. Whether CYP1A2 catalyzes arene oxide formation, and whether other cytochromes P450 or FMOs catalyze *N*-hydroxy-4-ABP formation, remains to be determined. It is conceivable, though quite unlikely from an evolutionary standpoint (rodents are ~80 million years diverged from humans, but the rat is only 17 million years diverged from mouse), that human and rat CYP1A2 participate in the N-hydroxylation of ABP whereas mouse CYP1A2 does not. In fact, using the baculovirus expression vector driving mouse and human CYP1A2, we have determined that N-hydroxylation of ABP is indeed catalyzed by mouse CYP1A2, but that by human CYP1A2 is more than three times greater, per unit of CYP1A2, semiquantitated by Western immunoblot (the authors unpublished data).

In summary, we had postulated that ABP-induced methemoglobinemia, a biomarker of exposure, might be greater in high-CYP1A2 workers than in low-CYP1A2 workers–and especially high in cigarette smokers because CYP1A2 is induced by cigarette smoke. This is especially clinically relevant because of more than 60-fold differences in basal liver CYP1A2 in human population studies. We therefore expected that ABP-induced methemoglobin formation would be higher in dioxin-induced as well as constitutive wild-type *Cyp1a2(+/+)* mice than in CYP1A2-null mice. We found exactly the opposite: the presence of CYP1A2 lessened methemoglobinemia, and dioxin pretreatment further lowered methemoglobin formation. In our mouse model, therefore, CYP1A2 does not appear to be primarily involved in the ABP-induced depletion of hepatic thiol levels nor in the production of ABP-elicited methemoglobinemia. Since these parameters are indicative of metabolic activation, the results suggest that other metabolic pathways must be considered in order to provide a reliable model for the biotransformation of ABP that leads to toxicity and carcinogenesis in mice.

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