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ACCELERATED COMMUNICATION

Piperonyl Butoxide and Acenaphthylene Induce Cytochrome P450 1A2 and 1B1 mRNA in Aromatic Hydrocarbon-Responsive Receptor Knock-Out Mouse Liver

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SUMMARY

It has been suggested that acenaphthylene (ACN), piperonyl butoxide (PBO) and other methylenedioxyphenyl (benzodioxole) compounds can function as aromatic hydrocarbon-responsive receptor (AHR)-independent inducers of the cytochrome P450 (CYP) 1A2 in mouse liver. Although much indirect evidence has supported this hypothesis, direct proof was lacking until the present study. PBO and ACN were used to examine the expression of CYP1A1, CYP1A2 and CYP1B1 in mouse liver. These three CYP isozymes are included in the AHR battery of proteins. In this study, AHR knock-out mice were dosed intraperitoneally with PBO (200 mg/kg) or ACN (100 mg/kg). Induction of hepatic CYP1A1 by PBO or ACN was not detected by northern blots. In contrast, both CYP1A2 and CYP1B1 mRNA, constitutively expressed at low levels in this tissue, were induced by each compound in the livers of AHR knock-out mice. In addition, the use of heterogenous nuclear RNA reverse transcription-polymerase chain reaction procedures revealed that the transcriptional activities of CYP1A2 were increased by PBO and ACN treatments. These results show that AHR-independent pathway(s) can be involved in induction of CYP1A2 and CYP1B1.

The aromatic hydrocarbon-responsive receptor (AHR) is an intracellular protein that mediates induction of a battery of genes including those encoding CYP1A1, CYP1A2, NAD(P)H: menadione oxidoreductase, a cytosolic "class 3" aldehyde dehydrogenase, a UDP glucuronosyltransferase, and a glutathione transferase (1). The AHR binds specific ligands such as TCDD or PAH and generates a heterodimeric transcription factor with the AHR nuclear translocator (2). In the nucleus, the AHR binds to a specific *cis*-activating enhancer (3) that promotes gene transcription (4, 5). The induction of CYP1A1, CYP1A2, and CYP1B1 reflects an increase in the rate of transcription of genes. In addition, CYP1A2 and CYP1B1 can be induced through post-transcriptional mechanisms (5).

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An $AHR^{-/-}$ mouse was recently developed that is homozygous for an *AHR* gene disrupted in exon 1 (6). In the wild-type mouse, CYP1A1 expression is inducible, whereas CYP1A2 expression is constitutive as well as inducible. In the $AHR^{-/-}$ mouse, CYP1A1 and CYP1A2 mRNA were not induced by TCDD and the constitutive expression of CYP1A2 was decreased by about 90% (6), thus demonstrating convincingly that AHR is associated with the expression of both of these genes.

MDP compounds, also known as benzodioxole compounds, such as safrole, isosafrole, and sesamex occur in various plants (7). PBO, a synthetic MDP, is used as an insecticide synergist with pyrethroid and carbamate insecticides (8–10). Some MDP compounds induce CYP1A1, CYP1A2 and CYP2B10 in the mouse, depending on the structures of the compounds (11–16). In early experiments, a number of MDP

ABBREVIATIONS: AHR, aromatic hydrocarbon-responsive receptor; ACN, acenaphthylene; $AHR^{-/-}$, aromatic hydrocarbon-responsive receptor knock-out; CYP, cytochrome P450; hnRNA, heterogenous nuclear ribonucleic acid; 3-MC, 3-methylcholanthrene; MDP, methylenedioxyphenyl; PAH, polycyclic aromatic hydrocarbon; PBO, piperonyl butoxide; RT, reverse transcription; PCR, polymerase chain reaction; TCDD, 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin; bp, base pair(s).

compounds were found to induce levels of CYP1A2 mRNA and protein without the induction of CYP1A1, which suggests the possibility of an AHR-independent mechanism(s) of CYP1A2 induction (13–16). PBO, isosafrole, and 2,2-dimethyl-5-*t*-butyl-1,3-benzodioxole were unable to displace TCDD and 3-MC from the AHR or 4 S binding protein *in vitro* (17, 18). In addition, MDP compounds induced CYP1A2 without any correlation with the aromatic hydrocarbon-responsive locus (19, 20). ACN is one of the prototypical tricyclic PAHs that do not bind competitively to the hepatic cytosolic AHR or 4 S binding protein of B6C3F1 mouse (21, 22). The tricyclic PAHs have been shown to induce CYP1A1 as well as CYP1A2, but the induction of CYP1A1 was only minimal in the mouse (21, 22). Therefore, both MDP compounds and tricyclic PAHs induce CYP1A2 by a mechanism different from TCDD and related AHR agonists, which are known to induce CYP1A1, CYP1A2, and CYP1B1 by a common AHR-dependent pathway. In this study, $AHR^{-/-}$ mice were treated with PBO and ACN to examine their effects on CYP1 family expression.

Experimental Procedures

Materials. Piperonyl butoxide was purchased from ChemService (West Chester, PA), acenaphthylene from Crescent (Hauppauge, NY), avian myeloblastosis virus reverse transcriptases from Boehringer Mannheim (Indianapolis, IN), and Taq DNA polymerases from Promega (Madison, WI).

Animal Treatments. Male $AHR^{-/-}$ mice (6) weighing 26–29 g were injected intraperitoneally with PBO (200 mg/kg) or ACN (100 mg/kg) dissolved in corn oil (100 μl/mouse) and killed 24 hr after injection by CO_2 asphyxiation. The tissues were snap frozen in liquid nitrogen and stored at -80° before use.

Northern Blots. cDNA probes to mouse CYP1A2 (23) and human β-actin (24) were purchased from American Type Culture Collection (Rockville, MD). pBluescript plasmids containing mouse CYP1B1 cDNA were a generous gift from Dr. Colin R. Jefcoate (Department of Pharmacology, University of Wisconsin-Madison) (25). The sequence of an oligonucleotide probe to mouse CYP1A1 was described earlier (15). Total RNA extraction, poly(A)$^+$ RNA fractionation and northern blots were performed as reported (15). Briefly, ^{32}P-labeled probes were generated by a random priming procedure. RNA was fractionated in formaldehyde-agarose gels and transferred to nylon membranes. The membranes were prehybridized, hybridized, and washed according to standard procedure before autoradiography to X-ray film.

hnRNA RT-PCR. RT-PCR on hnRNA has been used to detect transcriptional activity as a substitute for the nuclear run-on assay (26–31). Although the nuclear run-on assay measures newly produced mRNA, hnRNA RT-PCR gives hnRNA levels determined by both transcription and post-transcriptional processing. In contrast to northern blotting, both hnRNA RT-PCR and nuclear run-on assay are unaffected by mRNA stabilization or destabilization. Preliminary studies in this laboratory using 3-MC treated C57BL and DBA/2 mouse liver RNA samples confirmed the usefulness of this technique for the demonstration of increased transcription rates (data not shown). In the present study, hnRNA RT-PCR was performed to determine the change in the transcriptional activities of CYP1A2. Total RNA from liver was prepared as stated above. Total RNA samples were treated with RNaseH and then used in PCR to determine the genomic DNA contamination before further use. RT of the RNA samples was performed using random primers and avian myeloblastosis virus reverse transcriptase. The cDNA samples were used in PCR for CYP1A2 hnRNA cDNA using two primers from intron B and intron C, respectively (32). The PCR product, therefore, contains an exon region (3438–3555 bp). The upper primer spans 3233–3253 bp (5′-TGGGGTTATGGGAAAGAAGGG-3′), and the lower primer spans 3667–3646 bp (5′-CACACCTTGATCTTAGGGC AGG-3′). To maximize the linearity of PCR, the number of cycles was limited to 20 (33). After the PCR, each sample was probed with a CYP1A2 cDNA probe in Southern blots. RT-PCR and Southern blots for β-actin were also performed to determine the quality of total RNA.

Results and Discussion

The northern blot for CYP1A2 mRNA (Fig. 1) showed that the mRNA was expressed constitutively, although at a low level, in $AHR^{-/-}$ mouse as reported (6). PBO and ACN, which were suggested to be AHR-independent inducers of CYP1A2 (20, 21), increased the CYP1A2 mRNA levels in $AHR^{-/-}$ mouse liver through increased transcriptional activities of the gene (Fig. 2). Both constitutive and inducible expression of CYP1A2 in $AHR^{-/-}$ mouse liver provide direct proof of an AHR-independent pathway(s), which has been hypothesized from various indirect evidence (13–22, 34). Studies using Hep G2 cells transiently transfected with human *CYP1A2* 5′-regulatory region-fusion gene constructs showed that various sequences are responsible for constitutive (35) and inducible (36) expression. Several elements important for constitutive and inducible expression of CYP1A2 in Hep G2 cells span −2000 to −2600 bp (relative to transcription initiation sites) (35, 36). Some *cis*-elements regulate the constitutive expression of human CYP1A2; one of them contains the hepatic nuclear factor-1 binding site, supposedly giving liver specificity to this CYP. Two other xenobiotic response element-like

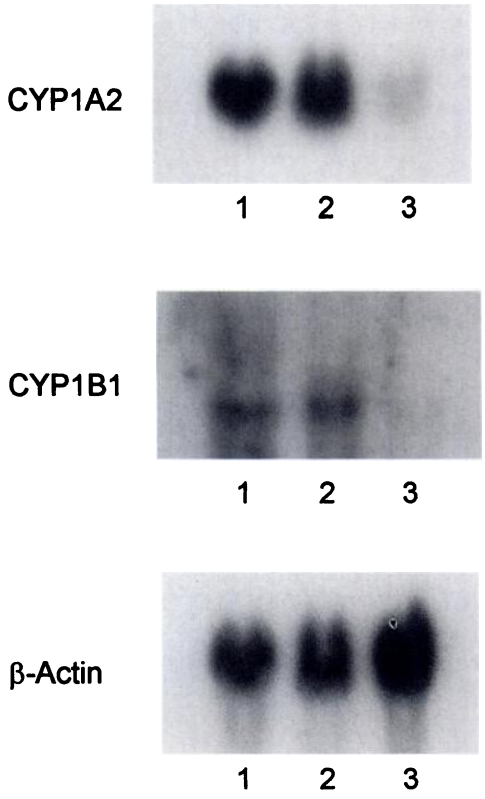

Fig. 1. Induction of CYP1A2 and CYP1B1 in $AHR^{-/-}$ mouse liver by acenaphthylene and piperonyl butoxide. Northern blots show hepatic CYP1A2, CYP1B1, and β-actin levels after 24-hr treatment. Ten micrograms of poly(A)$^+$ RNA were loaded in each lane. *Lane 1*, $AHR^{-/-}$ ACN (100 mg/kg); *lane 2*, $AHR^{-/-}$ PBO (200 mg/kg); *lane 3*, $AHR^{-/-}$ corn oil.

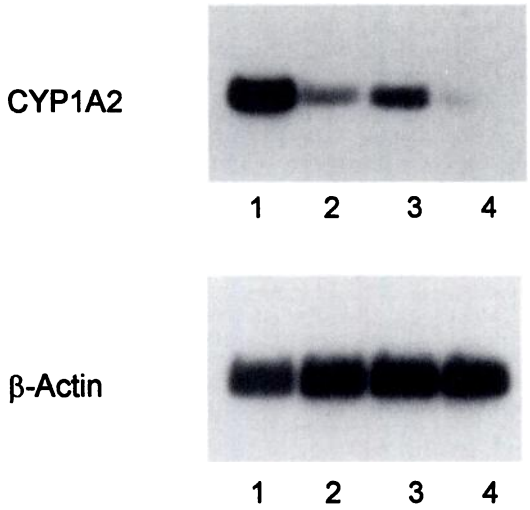

Fig. 2. Induction of CYP1A2 hnRNA in AHR$^{-/-}$ mouse liver by acenaphthylene or piperonyl butoxide. Southern blots for PCR product show CYP1A2 and β-actin hnRNA levels after 24-hr treatment. The size of PCR product for CYP1A2 hnRNA is 435 bp. *Lane 1,* C57BL strain -3-methylcholanthrene (50 mg/kg); *lane 2,* AHR$^{-/-}$ ACN (100 mg/kg); *lane 3,* AHR$^{-/-}$ PBO (200mg/kg); *lane 4,* AHR$^{-/-}$ corn oil.

sequences, presumably AHR-dependent, are responsible for CYP1A2 induction by 3-MC. Mouse *Cyp1a-2* sequences between −1843 bp and +52 bp were found to be insufficient for constitutive and TCDD-inducible expressions of the gene in mouse hepatoma cells (37). At this time, we do not have access to the AHR$^{+/+}$ and AHR$^{+/-}$ mice. Because results from previous studies indicated induction of CYP1A2 by a non-AHR mechanism as well as AHR mechanism (13–22, 34), we were primarily interested in confirming the non-AHR mechanism.

CYP1B1 was constitutively expressed in AHR$^{-/-}$ mouse livers, although the levels were very low (Fig. 1). Constitutive expression of CYP1B1 has also been shown in C57BL and DBA/2 mice livers.[1] Constitutive expression of CYP1B1 was readily detected in adrenal glands, ovaries, and testes of rats (38–40). CYP1B1 has also been shown to be expressed constitutively and to be induced by adrenocorticotropic hormone in cultured rat adrenal cells, probably through the mediation of cAMP (41). TCDD was shown to induce CYP1B1 in liver, lung, and kidney of rats (40), presumably mediated through AHR. In contrast, the current results show that CYP1B1 can also be induced in the absence of AHR (Fig. 1). These results suggest that CYP1B1 is regulated by more than one mechanism. The hnRNA RT-PCR for CYP1B1, however, was not performed because of the unavailability of its genomic DNA sequence.

In C57BL and DBA/2 mice, PBO was found to induce CYP1A1 only at high dose levels (400 mg/kg), with induction being much greater in C57BL than DBA/2 (20).[1] Likewise, ACN induced CYP1A1 mRNA in B6C3F1 mice only at high doses (21, 22). Neither PBO nor ACN was demonstrated to bind to AHR (9 S) or glycine *N*-methyltransferase (4 S) (42), mediators of the induction of CYP1A1, by use of TCDD binding (19, 21). In addition, the current study shows that CYP1A1 induction was undetectible by both compounds in AHR$^{-/-}$ mice (data not shown). Based upon these results, we suggest that PBO and ACN induce CYP1A1 through inefficient activation of AHR in the inbred strains stated above. It should be noted that benzimidazoles induce CYP1A through ligand independent activation of AHR in rabbit (43).

PBO, a methylenedioxyphenyl compound, and ACN, a tricyclic PAH, induced CYP1A2 and CYP1B1 noncoordinately with CYP1A1. The induction characteristics of these two classes of compounds must be based upon their unique chemical structures: methylenedioxyphenyl ring structures (13–15, 34, 44) and tricyclic structures (21–22), respectively. Therefore, it can be assumed that MDP compounds and tricyclic PAHs activate endogenous signaling pathway(s) to induce both of these CYPs. Further studies are expected to determine whether CYP1A2 and CYP1B1 induction by either class of compounds is caused by the amplification of constitutive expression or by a novel mechanism(s) of induction.

[1] D.-Y. Ryu, P. E. Levi, and E. Hodgson, manuscript in preparation.

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