ACCELERATED COMMUNICATION

Aspartate Aminotransferase Generates Proagonists of the Aryl Hydrocarbon Receptor

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ABSTRACT
The aryl hydrocarbon receptor (AHR) binds planar aromatic compounds and up-regulates the transcription of a battery of xenobiotic-metabolizing enzymes. To identify proteins involved in the biosynthesis of endogenous AHR ligands, we screened extracts of various mouse tissues for AHR signaling activity. We found heart extract to activate AHR and identified the active component to be the enzyme aspartate aminotransferase (EC 2.6.1.1). We demonstrate that this transaminase can activate AHR signaling by converting L-tryptophan to indole-3-pyruvate. In turn, indole-3-pyruvate spontaneously reacts in aqueous solution to form a large number of compounds that act as agonists of AHR. Tyrosine and the serotonin-precursor 5-hydroxytryptophan also activate AHR signaling in combination with aspartate aminotransferase, suggesting that 4-hydroxyphenylpyruvate and 5-hydroxyindolepyruvate also act as pro-agonists of AHR. This study demonstrates that the known tryptophan metabolic-intermediate indole-3-pyruvate is a pro-agonist of AHR that reacts in aqueous solution to form a variety of AHR agonists.

The aryl hydrocarbon receptor (AHR) is a ligand-activated member of the Per/ARNT/Sim (PAS) superfamily of transcriptional regulators (Gu et al., 2000). Known xenobiotic ligands of the Ah receptor include polycyclic aromatic hydrocarbons, such as benzo[a]pyrene, and halogenated aromatic hydrocarbons, such as the polychlorinated dioxins and polychlorinated biphenyls (Sawyer and Safe, 1982; Poland et al., 1985; Safe et al., 1985). Upon ligand binding, AHR translocates from the cytosol to the nucleus, dimerizes with another PAS protein known as ARNT, and this heterodimer binds to DNA sequences known as dioxin responsive elements (DREs) and activates transcription (Hankinson, 1995; Schmidt and Bradfield, 1996; Whitlock, 1999). The result of this signaling pathway is the up-regulation of a battery of xenobiotic-metabolizing enzymes (XMEs), which includes the cytochrome P450 monooxygenases 1A1 (CYP1A1), 1A2 (CYP1A2), and 1B1 (CYP1B1) (Nebert et al., 1972). Because many AHR agonists are substrates for these XMEs, they are ultimately metabolized and excreted as more polar derivatives.

The AHR has been shown to play an important role in normal development (McDonnell et al., 1996; Schmidt et al., 1996). Although viable, AHR knockout mice display a slower growth rate, decreased fertility, and a reduced liver size. These phenotypes seem to be related to the fact that adult AHR null mice have an altered vascular architecture (Lahvis et al., 2000). The developmental abnormalities in AHR-null animals indicate that this receptor is required during development and that AHR may be activated by an endogenous ligand in the course of normal development.

Our hypothesis is that the physiological role of the AHR is to regulate the metabolism of endogenously generated compounds that may be deleterious to the cell. The goal of this study was to identify enzymes that are involved in the production of AHR agonists. By identifying components of this pathway, we may ultimately identify the small molecule ligands that are important during development.

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; PBS, phosphate-buffered saline; XME, xenobiotic-metabolizing enzymes; BNF, β-naphthoflavone; DMSO, dimethyl sulfoxide; cAST, cytosolic aspartate aminotransferase; I3P, indole-3-pyruvate; HPP, 4-hydroxyphenylpyruvate; 5-HT, 5-hydroxy-L-tryptophan; PAGE, polyacrylamide gel electrophoresis; AST, aspartate aminotransferase; mAST, mitochondrial aspartate aminotransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; HPLC, high-performance liquid chromatography.
Materials and Methods

Chemicals. Porcine cytosolic aspartate aminotransferase (cAST) and rat hemoglobin (both from Sigma-Aldrich, St. Louis, MO) were dissolved in phosphate-buffered saline (PBS), deaslated with a PD-10 column (Amersham Biosciences Inc., Piscataway, NJ), and stored at −80°C. All amino acids (Sigma-Aldrich), indole-3-pyruvic acid (13P, 99% purity), 4-hydroxyphenylpyruvic acid (HP, 98% purity), 5-hydroxy-tryptophan, α-ketoglutarate, oxaloacetate, indole-3-acetate, β-naphthoflavone (BNF), bilirubin, and hemin were of the highest purity available and were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was the solvent for BNF, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and hemin. Ethanol was the solvent for 13P, HPP, and indole-3-acetate stock solutions.

Cell Lines, Growth Conditions, and Plasmids. All cell lines were grown in Dulbecco’s modified Eagle’s medium with high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM l-glutamine, minimal essential medium nonessential amino acids (Invitrogen), and 10 mM HEPES, pH 7.5. Cultures were maintained at 7.5% CO2 in a humidified incubator, 1 mM sodium pyruvate, 1 mML-glutamine, minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum modified Eagle’s were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The rat hepatoma cell line BP8 contains a stably integrated luciferase reporter gene driven by the DREs found within the CYP1A1 promoter (Postlind et al., 1993). All reporter gene assays were carried out 15 to 20 h after treatment of the cells with extracts or purified compounds. To identify the fractions that activated AHR signaling from the various protein purification steps, aliquots of each fraction were applied to PD-10 desalting columns to exchange the buffer with PBS and 100 µl was applied to 101L cells grown in 500 µl of media in 24-well plates.

Results and Discussion

Mouse Hearts Contain a Factor that Activates AHR. In our search for endogenous ligands of the AHR, we screened extracts of various murine tissues for factors that would activate this receptor. We used a cell-based reporter system to identify agonist activity in the tissue extracts (Postlind et al., 1993). In our initial screening, we incubated minced mouse tissues in PBS overnight and screened the aqueous extracts for their ability to activate the AHR reporter system. Although we observed weak activity in a number of tissues, heart extract consistently contained the greatest amount of activity (Fig. 1A). To estimate the molecular size of the factor, we concentrated the heart extract using an ultrafiltration device with a 50-kDa molecular mass cut-off ultrafiltration device, and loaded onto a HiPrep 16/60 Sepharose S200 gel filtration column (Amersham Biosciences) that had been preequilibrated with PBS, and proteins were eluted with PBS. Protein fractions were collected by SDS-PAGE and visualized with SYPRO Ruby stain (Bio-Rad, Hercules, CA). The major protein band in the fraction containing the activity peak was excised from the gel and trypsinized, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry at the University of Wisconsin Biotechnology Center (Madison, WI). Masses of the peptide fragments were used to search the protein databases using the computer program MS-Fit (University of California, San Francisco, San Francisco, CA) (Clauser et al., 1999).

Separation of 13P Derivatives. Stock solutions of 100 mM 13P in ethanol were diluted into PBS to a final concentration of 5 mM and allowed to incubate protected from light at room temperature for various periods of time. Solutions of 13P were fractionated by passage of 5 mL of 5 mM 13P in PBS over a C18 SPICE solid-phase extraction cartridge (Analytech, Newark, DE), washed with 5 mL of water, and eluted with 3 mL of methanol. Methanol elutions were concentrated by evaporation under nitrogen and dissolved in smaller volumes of methanol. Compounds in the methanol elutions were separated by HPLC onto a C18 column (Alltech Associates, Deerfield, IL) using a 5-min elution with 0.05% triethylamine in water, followed by a 100-min linear gradient of 0.05% triethylamine in water to 100% methanol with a 1 mL/min flow rate. Eluted compounds were detected by monitoring the absorbance at 280 nm (A280). To measure inducing activity, 0.25-mL fractions were collected, and an aliquot of each fraction was added to 101L cells and assayed as described above.

CYP1A1 Inhibition Assay. Fourteen units of purified CYP1A1 microsomes (Sigma-Aldrich) were diluted in 162 μl of 25 mM 4-morpholinepropanesulfonic acid, pH 7.5/0.025% sodium azide/1 mM EGTA/10% glycerol. Three microliters of 0.1 mM ethoxyresorufin (Sigma-Aldrich) in ethanol and 20 μl of the various competitor compounds were added to each reaction. Competitor compounds were diluted into PBS from 100-mM ethanol stocks. Where necessary, 13P solutions that had been incubated at room temperature in the dark for 20 h, were re-diluted with 20 μl of 5 mM β-nicotinamide adenine dinucleotide phosphate-reduced tetrasodium salt, and the accumulation of the hydroxyresorufin was monitored at room temperature in a microplate fluorometer using an excitation wavelength of 510 nm and an emission wavelength of 590 nm.
To confirm that the factor activated transcription via the AHR, we monitored the response of a DRE-driven reporter in cells with and without the receptor. Rat BP8 cells, which lack endogenous AHR, were used as our receptor-deficient cell line, and BP8 cells that had been transiently transfected with AHR were used as our receptor-positive cell line. Cells were either exposed to 40 mM HEPES, pH 7.5, or the concentrated heart extract in HEPES buffer. For use as controls, we included 20 nM TCDD or the solvent DMSO. Expression of the luciferase reporter gene was quantified after an 18-h incubation period (Fig. 1B). None of the experimental treatments induced transcription from the DRE promoter without the expression of AHR. In cells transfected with the AHR expression construct, both TCDD and the concentrated heart extract increased reporter gene activity, indicating that AHR is necessary to mediate the transcriptional response to the heart extract.

Purification and Identification of Aspartate Amino-transferase as the Active Component. In preliminary biochemical studies of the heart extract, a number of important observations were made that directly led to the success of the purification strategy (data not shown). First, whenever a high-resolution purification step was used, very little activity was recovered. This low recovery was in contrast to our data indicating that the activity in the heart extract was quite stable to heat, pH, and salt. This observation suggested that multiple components of heart extract were needed in combination for the AHR-inducing activity. Second, in various chromatographic separations, we observed that the inducing activity always eluted in fractions adjacent to fractions that had a deep red color. Moreover, when we combined fractions containing the red color with fractions that had low activity, we found that activity was increased significantly. Because this red protein was very abundant, it was readily purified and identified by MALDI-TOF mass spectrometry to be β-hemoglobin. This led us to add commercially available rat hemoglobin (0.5 mg/ml, final concentration) in combination with the column fractions when assaying for AHR-inducing activity. This addition restored the activity of our fractions and allowed us to purify the active component to near homogeneity.

We developed a protein-purification scheme to isolate the active component of heart extract (Fig. 2A). We followed the purification of the active component by performing SDS-PAGE on active fractions from each chromatographic step. We found the active fractions from the final gel-filtration step contained only one major protein (Fig. 2, B and C). To identify this protein, the protein band was excised from the gel and trypticized, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry (Fig. 2D). This procedure identified this major protein as cAST (also known as aspartate transaminase or glutamate oxaloacetate transaminase-I; EC 2.6.1.1). This enzyme is best known to catalyze the transfer of an amino group from aspartate to α-ketoglutarate producing oxaloacetate and glutamate (Fig. 2E). Two isoforms of this aminotransferase are present in cells, one in the cytosol (cAST), and one in the mitochondria (mAST) (Cooper and Meister, 1985). These enzymes are known to play a role in gluconeogenesis and in the malate-aspartate shuttle, which transports reducing equivalents from the cytosol to the mitochondria (Cooper and Meister, 1985). Heart tissue is known to express extremely high levels of AST, and elevated levels of AST in the serum are used as a clinical indication of cardiac damage (Schmidt and Schmidt, 1985).

To confirm that AST was the source of the activity in our heart extracts, we obtained commercially available porcine CAST and found that it activated AHR signaling in a hemoglobin-dependent manner (Fig. 3A). Although our purification only identified cAST as the active component released from heart tissue, we also tested the commercially available porcine mAST and found that this protein was just as active as cAST in our AHR reporter assay system (data not shown).
Exogenous Iron Greatly Enhances the Signaling Activity of cAST. To understand how hemoglobin was enhancing the AHR-activating activity, we performed a number of biochemical experiments. First, we found that other heme-containing proteins were also active, because both bovine hemoglobin and horse myoglobin activated AHR signaling with cAST (data not shown). Because these proteins share an iron-containing heme moiety, we also tested hemin and FeCl₃ and found that both of these compounds activated AHR signaling with cAST (Fig. 3A). Finally, the addition of the heme product bilirubin did not increase cAST signaling, and the addition of a 10-fold excess of the iron chelator deferoxamine mesylate abolished activation by hemoglobin, hemin, and FeCl₃ (data not shown). Taken together, these data suggest that it is the iron which is contained in these compounds that acts with cAST to activate AHR signaling.

The Enzymatic Activity of cAST Activates AHR. With the identification of cAST came the initial hypothesis that one or more of the products of its enzymatic reaction is related to the activation of AHR. To test this, the substrates aspartate, α-ketoglutarate, oxaloacetate, and glutamate were added to 101L cells at a final concentration of 1 mM and tested for their ability to activate AHR in the absence of cAST. None of these compounds induced AHR signaling when added to the cell-culture media (Fig. 3B) and did not activate AHR signaling when added to cells in combination with hemoglobin (data not shown). The addition of α-keto-glutarate and oxaloacetate, the amino group acceptor compounds, enhances the AHR signaling of cAST, whereas the amino donor compounds aspartate and glutamate, either slightly reduce the signaling or have no effect, respectively (Fig. 3B). Taken together, these data suggest that it is the enzymatic activity of cAST that is stimulating the AHR pathway; however, aspartate and glutamate are not the amino donor compounds.

To identify the amino donor compounds that were the source of the AHR signaling activity, we screened each common amino acid for its ability to activate AHR signaling in a cAST-dependent manner (Fig. 3C). Each amino acid was added to the cell-culture medium (0.4 mM final concentration) in combination with 0.4 mM α-ketoglutarate and 0.2 mg/ml of rat hemoglobin. None of the amino acids activated AHR signaling in the absence of cAST. When added to cells with 13 U/ml porcine cAST, we consistently observed increased signaling in treatments containing tryptophan or tyrosine, with tryptophan providing the greatest increase in signaling. The hydroxylated derivative of tryptophan, 5-hydroxy-L-tryptophan (5-HT), was also found to activate AHR signaling in a cAST-dependent manner, with 5-HT being nearly as active as tryptophan when added to 101L cells in combination with cAST, α-ketoglutarate, and hemin (Fig. 3D).

Given that cAST is known to use aromatic amino acids as alternative substrates (Miller and Litwack, 1971; Minato-
gawa et al., 1976), we diagramed the transamination reactions using tryptophan, tyrosine, and 5-HT as amino donor compounds (Fig. 3E). Transamination reactions with tryptophan, tyrosine, and 5-HT as amino donor compounds generate I3P, HPP, and 5-hydroxyindolepyruvate, respectively (Cooper and Meister, 1985). When we added 500 μM I3P or HPP to the cell-culture system, both compounds activated AHR signaling, with I3P being much more active than HPP (Fig. 3F). 5-Hydroxyindolepyruvate was not tested for activity because it is not commercially available. The signaling activities of both I3P and HPP were greatly enhanced by the addition of 100 μM FeCl₃ to the cells. Hemoglobin and hemin were also found to enhance the activity of I3P and HPP (data not shown), suggesting that the iron requirement of cAST in our activity assay occurs after the enzymatic reaction catalyzed by cAST. Because I3P activated AHR signaling much more than HPP, we focused the remainder of our studies on I3P.

### I3P Spontaneously Reacts in Aqueous Solution to Form AHR Agonists

Although stock solutions of I3P in ethanol seem relatively stable, on dilution into PBS, I3P solutions begin to turn pink within minutes of dilution and become red-orange after an overnight incubation at room temperature (data not shown). In an effort to determine whether this aqueous instability was related to AHR signaling activity, aqueous solutions of I3P were incubated for various times at room temperature, protected from light, and screened for AHR signaling activity. We observed a rapid increase in AHR signaling activity, with a maximum accumulation of activity between 10 and 48 h of incubation (Fig. 4A). When oxygen was bubbled through aqueous solutions of I3P, we observed a more rapid accumulation of colored products and AHR signaling activity (data not shown), suggesting that oxidative chemical reactions are involved in generating AHR agonists from I3P. Because the addition of FeCl₃ would increase the oxidative environment of the cell-culture media (Ercal et al., 2001), the synergistic role of iron in our activity assays may be explained by an accelerated oxidation of I3P and I3P derivatives mediated by iron. These experiments led us to conclude that I3P is itself not active as an AHR agonist, but that it spontaneously converts to one or more agonist compounds in aqueous solution.

### I3P and HPP Synergistically Interact

Although HPP has much less inducing activity than I3P, HPP synergistically interacts with I3P to activate AHR signaling. Exposure of cells to a final concentration of 12.5 μM of both I3P and HPP activated AHR signaling substantially more than did exposure to 25 μM of I3P alone in the presence of 100 μM FeCl₃ (Fig. 4B). This observation suggests that I3P and HPP are either reacting together to form increased quantities of the I3P-derived agonist(s) or reacting together to generate one or more agonist compounds that are structurally distinct from the agonist(s) derived from I3P alone.

### I3P Derivatives Bind the Active Site of CYP1A1

We predicted that, like many of the polycyclic aromatic compounds that activate AHR, ligands of the AHR would also be metabolized by the battery of XMEs that are regulated by AHR. To determine whether I3P derivatives interact with the active site of the CYP1A1 protein, we measured the ability of I3P derivatives to inhibit CYP1A1 enzymatic activity with the substrate ethoxyresorufin. Various amounts of either preincubated or freshly made I3P aqueous solutions were added to microsomes containing purified CYP1A1, and ethoxyresorufin-deethylase activity was measured. We observed that the preincubated I3P had approximately 10-fold greater inhibitory activity than the I3P that was diluted into PBS and assayed immediately (Fig. 4C). The compound indole-3-acetate, which is not an inhibitor of CYP1A1 (Heath-Pagliuso et al., 1998), was included as a negative control. We plotted CYP1A1 kinetic data in a Lineweaver-Burk plot to determine the nature of this competitive interaction (Fig. 4D). This analysis led us to conclude that the I3P derivatives...
act as a “mixed competitor” in our assays, suggesting that the binding of I3P derivatives occurs at enzyme sites involved in both substrate binding and catalysis. Thus, the compounds generated from I3P not only induce transcription of the CYP1A1 gene via AHR activation, they also bind to the active site of the CYP1A1 protein as well. This is consistent with our hypothesis that these compounds may act as endogenous agonists of AHR and induce their own metabolism.

**Multiple Agonist Compounds Are Generated from I3P.** To determine the number of biologically active products that were produced from I3P, we separated the aqueous reaction products by solid-phase extraction onto C18 resin. This separation generated three crude fractions: compounds that did not bind the C18 resin, compounds that eluted with water, and compounds that eluted with methanol. Aliquots of each of these fractions were added to 101L cells to determine which fraction contained the most activity (Fig. 5A). When solutions of I3P that were incubated for 20 h were separated, large amounts of activity were observed in all three fractions. These results indicate that the activity generated during the incubation is caused not by the formation of a single I3P derivative with agonist activity but by multiple compounds with a wide range of hydrophobic character.

In an effort to determine the complexity of the I3P derivatives that activate AHR signaling, we resolved the more hydrophobic derivatives of I3P that eluted from the C18 resin with methanol. We separated the compounds in this mixture by reverse-phase HPLC and assayed each fraction in our cell-culture system. We compared HPLC profiles of the compounds from aqueous solutions of I3P that were incubated at room temperature for 20 h as well as solutions of I3P that were subjected to solid-phase extraction immediately after dilution into PBS, designated time 0 h. The HPLC profile of the compounds in the 0-h incubation demonstrates that derivatives of I3P are beginning to form even in the short period during which I3P is exposed to an aqueous environment (Fig. 5B). However, these early products had little AHR signaling activity (Fig. 5C). The major product in this mixture was identified as indole-3-carboxaldehyde (data not shown), which is known not to be an agonist of the AHR (Bradfield and Bjeldanes, 1987). The HPLC profile of the products of the 20-h incubation indicated that I3P converts to dozens of distinct compounds (Fig. 5D). What was striking about the activity profile of column fractions from the 20-h products was the large number of peaks of activity. The activity profile was almost as complex as the \( A_{280} \) trace (Fig. 5E), indicating that a large number of I3P derivatives are AHR agonists. As a control to ensure that our HPLC analysis and assay system was not responsible for the complex profile, we separated a stock solution of BNF on the column and observed a single major \( A_{280} \) and activity peak (data not shown).

**Conclusions**

The importance of naturally occurring and endogenous 3-substituted indoles is an emerging theme in AHR biology. In particular, the data support the concept that certain metabolites of 3-substituted indoles undergo spontaneous condensation to form a spectrum of potent AHR agonists at physiological conditions. In previous studies, we have shown that indole-3-carbinol, an indole compound present in foods derived from plants of the *Brassica* genus, undergoes acid catalyzed condensation reactions in the acidic environment of the stomach to form a number of linear and cyclic dimers and trimers, including the high-affinity agonist indolo[3,2-b]carbazole (Bjeldanes et al., 1991; Grose and Bjeldanes, 1992). A number of previous reports have shown that tryptophan derivatives can activate AHR signaling (Rannug et al., 1987;...
Helferich and Denison, 1991; Miller, 1997; Heath-Pagliuso et al., 1998; Wei et al., 2000). In fact, it has been suggested that an unknown tryptophan-derived endogenous ligand is generated in mouse Hepa-1 cell lines, which both induces CYP1A1 transcription and is metabolized by the CYP1A1 protein (Wei et al., 2000). We demonstrate in this study that the known tryptophan metabolite I3P spontaneously reacts to form a family of AHR agonist compounds and that at least some of the I3P derivatives interact with the active site of the CYP1A1 protein. As an analogy to the concept of a procarcinogen from the field of chemical carcinogenesis, we refer to I3P as a “proagonist” compound because I3P does not activate AHR itself, but it is derivatives of I3P that activate AHR.

Although the structural determination of the ultimate agonist compounds derived from these proagonists will be an important achievement, it will be a time-consuming undertaking. Because I3P has the potential to react with other compounds within the cell, our identification of the most hydrophobic AHR agonists derived solely from I3P (Fig. 5E) is probably an underestimate of the complexity of compounds that can act as endogenous agonists of AHR. For example, the synergistic interaction of I3P and HPP in the activation of AHR signaling may be caused by the formation of a different spectrum of AHR agonist compounds beyond those generated from I3P alone. In addition, because 5-hydroxy-L-tryptophan activates AHR in combination with AST, we predict that the proagonist 5-hydroxyindolopruvate is generated, and it is likely that at least some of the AHR agonists generated from this compound are different from those generated from I3P. Interestingly, a product of I3P and cysteine may have already been identified from lung tissue (Song et al., 2002). Taken together, these data support the proposal that there is no single endogenous ligand of AHR, but that the AHR has evolved to respond to many different hydrophobic by-products of normal metabolism (Denison et al., 2002).

These results also highlight issues relevant to clinical pharmacology. For example, I3P has recently been used in a variety of clinical trials to reduce anxiety and induce sleep, with doses as large as 300 mg administered orally to volunteers (Politi et al., 1999). Because we found acidified solutions of I3P to generate agonist activity much more rapidly than solutions of I3P at neutral pH (data not shown), oral administration of I3P would expose I3P to the acidic stomach environment and would introduce a great potential of generating potent AHR agonists within the gastrointestinal tract. Exposure to such AHR agonists may unexpectedly alter drug metabolism in I3P recipients via the induction of the xenobiotic-metabolizing enzyme battery. In fact, because exposure to AHR agonists has been shown to increase serum tryptophan levels (Unkila et al., 1998), it is possible that some of the pharmacological effects of the I3P administration may be ultimately attributed to the AHR agonists generated from I3P.

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References


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