Activation of the Ah Receptor by Tryptophan and Tryptophan Metabolites†

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ABSTRACT: The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates many of the biological and toxicological actions of a variety of hydrophobic natural and synthetic chemicals, including the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin). A variety of indole-containing chemicals, such as indole-3-carbinol, indolo[3,2-*b*]carbazole, and UV photoproducts of tryptophan (TRP), have previously been identified as ligands for AhR. Here we have examined the ability of endogenous metabolites of tryptophan (TRP) to bind to and activate AhR in vitro and in cells in culture. Although hydroxylated TRP metabolites were inactive, two metabolites, namely tryptamine (TA) and indole acetic acid (IAA), were shown to be AhR agonists. Not only do TA and IAA bind competitively to AhR, but they also can stimulate AhR transformation and DNA binding and induce expression of an AhR-dependent reporter gene in cells. In addition to being an AhR ligand, TA is also a competitive substrate for cytochrome P4501A1, a well-characterized AhR- and TCDD-inducible gene product. Although these compounds are relatively weak ligands, compared to TCDD, they represent some of the first endogenous hydrophilic AhR agonists identified to date.

The Ah receptor (AhR)[1] is a basic helix−loop−helix-containing transcription factor which activates gene expression in a ligand-dependent manner (*1*−*3*). Exposure to TCDD, the prototypical and most potent AhR ligand, results in a wide variety of species- and tissue-specific toxic and biological responses, including tumor promotion, thymic involution, immune suppression, endocrine disruption, wasting, lethality, and induction of gene expression, and many of these effects have been shown to be AhR-dependent (*4*, *5*). Mechanistically, the AhR functions in a manner somewhat similar to that of the glucocorticoid receptor, although they are clearly members of distinct families of transcription factors (*1*−*3*). Following ligand (TCDD) binding, the cytosolic TCDD−AhR complex undergoes transformation, during which it dissociates from two molecules of hsp90 (a heat shock protein of 90 kDa) and at least one additional protein (*6*−*8*), it translocates into the nucleus (*9*, *10*), and following its association with at least one nuclear factor, ARNT (AhR nuclear translocator) protein, it is converted into its high-affinity DNA binding form (*1*−*3*, *11*, *12*). The binding of the transformed heteromeric AhR complex to its specific DNA recognition site, the dioxin responsive element (DRE), leads to chromatin and nucleosome disruption, increased promoter accessibility, and increased rates of transcription of the adjacent gene (*3*, *11*, *13*).

Currently, ligands for the AhR are known to include a variety of toxic and carcinogenic halogenated aromatic hydrocarbons (such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, and other chemicals), polycyclic aromatic hydrocarbons (such as benzo[*a*]pyrene, benzanthracene, dibenz[*a*,*h*]anthracene, etc.), heterocyclic amines and a variety of indole-containing compounds (*4*, *14*−*16*). Although the ability of a number of dietary chemicals to bind to and/or activate the AhR or AhR-dependent gene expression initially suggested that the natural ligand(s) for the AhR might be dietary in origin (*14*, *17*−*19*), several recent reports have indirectly indicated that an endogenous physiological ligand(s) for the AhR exists. An endogenous AhR ligand has been suggested both from studies using cells in culture, where induction of AhR-dependent gene expression has been observed in the absence of added exogenous ligand (*20*−*23*), and the occurrence of developmental defects in AhR knockout mice (*24*, *25*). Interestingly, the recent identification of bilirubin as a weak endogenous ligand for AhR indicates not only that the AhR can be activated by an endogenous chemical but also that it can be activated by a substance with physical and chemical properties distinct from

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[1] Abbreviations: AhR, aromatic hydrocarbon receptor; DMSO, dimethyl sulfoxide; DRE, dioxin responsive element; DTT, dithiothreitol; EC_{50}, effective concentration at 50%; EROD, ethoxyresorufin *O*-deethylase; HEDG, 25 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol; IC_{50}, chemical concentration at 50% inhibition; IAA, indole acetic acid; 5-OH-TA, 5-hydroxytryptamine, serotonin; 5-OH-TRP, 5-hydroxytryptophan; MEL, melatonin; 5-MO-TA, 5-methoxytryptamine; TCDF, 2, 3, 7, 8-tetrachlorodibenzofuran; TCDD, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin; TA, tryptamine; TRP, tryptophan. In this report, we have defined transformation as the process by which the TCDD−AhR complex is converted into a form(s) which can bind to DNA with high affinity.

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known high-affinity AhR ligands (*26*). Although the endogenous ligands responsible for the effects observed in cell culture and knockout mice remain to be identified, given the promiscuous nature of ligand binding exhibited by AhR (*14*), it would not be surprising if more than one endogenous ligand for AhR exists.

Over the past 10 years, a number of laboratories have demonstrated that some plant-derived products, such as oxidized carotinoids (*19*), and indole-containing chemicals, namely indole-3-carbinol and its acidic condensation product indolo[3,2-*b*]carbazole as well as rutacarpine alkaloids (*17*, *18*), are AhR ligands and/or AhR agonists. In addition, conversion of tryptophan (TRP) into AhR ligands by enteric bacteria (*27*) and UV irradiation (*28*, *29*) has been reported. These data, combined with the fact that TRP can be metabolically converted into several endogenous signaling factors [i.e., serotonin (5-hydroxytryptamine, 5-OH-TA), tryptamine (TA), melatonin (MEL), and others (*30*−*34*)], led us to examine whether TRP or any of its natural metabolites could act as an AhR ligand(s). While this paper was in preparation, the ability of TRP and its metabolites to activate AhR-dependent gene expression in a recombinant yeast system was reported (*35*). However, neither the ability of these chemicals to directly stimulate AhR transformation and DNA binding nor their ability to activate the AhR-dependent signaling pathway in mammalian cells was examined. These are important considerations, especially given the fact that the ligand binding specificity of AhR expressed in yeast is not identical to that of mammalian cells (*36*, *37*). In this report, we have examined the ability of TRP and various TRP metabolites to directly interact with and activate AhR in vitro and in Hepa1c1c7 cells in culture, and demonstrate that two metabolites, namely TA and IAA, and to a lesser extent TRP, can competitively bind to AhR, induce AhR transformation and DNA binding, and AhR-dependent activation of gene expression.

MATERIALS AND METHODS

Chemicals. TCDD, TCDF, and [^{3}H]TCDD (37 Ci/mmol) were obtained from S. Safe (Texas A&M University, College Station, TX). [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) and DMSO from Aldrich (St. Louis, MO). TRP (L and D forms) and TRP metabolites [TA-HCl, IAA, 5-OH-TA, 5-hydroxytryptophan (5-OH-TRP), 5-methoxytryptamine (5-MOTA), and MEL] were from Sigma (St. Louis, MO) and were greater than 98% pure.

Animals and Preparation of Cytosol. Male Hartley guinea pigs (250−300 g), obtained from Charles River Breeding Laboratories (Wilmington, DE), and male Sprague-Dawley rats (200 g) from Charles River Wiga GmbH (Sulzfeld, Germany) were exposed to 12 h of light and 12 h of dark daily and were allowed free access to food and water. Guinea pig hepatic cytosol was prepared in HEDG buffer [25 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] as previously described (*38*) and was stored at −80 °C until it was used. Protein concentrations were determined by the Bradford method (*39*), using bovine serum albumin as the standard.

Cell Culture and Preparation of Nuclear Extracts. Nuclear extracts were prepared from confluent plates of mouse hepatoma (hepa1c1c7) cells which had been treated with the indicated chemical for 1 h at 37 °C as we have previously described (*40*). The resulting "crude" nuclear extracts were stored frozen at −80 °C until they were used and protein concentrations determined as described above.

Synthetic Oligonucleotides. A complementary pair of synthetic oligonucleotides containing the sequences 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATC-CGGAGTTGCGTGAGAAGAGCCA-3′ [corresponding to the AhR binding site of DRE3 (*11*) and designated as the wild-type DRE oligonucleotide] and 5′-GATCTGGCTCT-TCTCAC<u>A</u>CAACTCCGGATC-3′ and 5′-GATCCGGAGT-TG<u>T</u>GTG<u>A</u>GAAGAGCCA-3′ [identical to the wild-type DRE oligonucleotide but containing a single substitution (underlined) within the DRE core consensus sequence which disrupts binding of the transformed TCDD−AhR complex (*38*) and designated as the mutant DRE (mDRE) oligonucleotide] were synthesized, purified, annealed, and radiolabeled with [γ-^{32}P]ATP as described (*11*).

Ah Receptor Ligand Binding Analysis. Specific binding of [^{3}H]TCDD to the cytosolic AhR was measured by sucrose gradient centrifugation as described (*38*). Guinea pig cytosol (5 mg/mL) was incubated with 10 μM [^{3}H]TCDD in the presence of 1 μM TCDF, 2 mM TA, 2 mM IAA, or carrier solvent (20 μL/mL of DMSO for TCDF, water for TA, and ethanol for IAA) for 1 h at 4 °C. After the samples were treated with dextran-coated charcoal to remove unbound and loosely bound radioligand, they were subjected to centrifugation in 10 to 30% sucrose (v/v) gradients for 2 h at 65 000 rpm in a Beckman VTi65.2 rotor at 4 °C. Following centrifugation, the gradients were fractionated and the radioactivity present in each fraction was determined by liquid scintillation. The specific binding of [^{3}H]TCDD was determined by subtracting the radioactivity present in each fraction of a gradient containing [^{3}H]TCDD and TCDF (nonspecific binding) from the radioactivity in the corresponding fractions from a gradient containing [^{3}H]TCDD alone (total binding). Competitive displacement by TA or IAA was determined as above, except that 2 mM TA or IAA was used in place of TCDF.

Gel Retardation Analysis. DNA binding analysis of cytosolic AhR complexes transformed in vitro was carried out as we have described in detail (*41*, *42*). Briefly, guinea pig cytosol (16 mg of protein/mL) was incubated with TCDD (20 nM), TRP or a TRP metabolite at the indicated concentration, or carrier solvent [DMSO, water, or ethanol (20 μL/mL)] for 2 h at 20 °C, followed by gel retardation analysis. DNA binding of nuclear AhR complexes isolated from cells which had been incubated with TCDD (1 nM), TRP or a TRP metabolite at the indicated concentration, or carrier solvent (20 μL/mL DMSO, water, or ethanol) was carried out as previously described (*40*). The amount of ^{32}P-labeled DRE specifically bound in the TCDD-inducible protein−DNA complex was determined using a Molecular Dynamics phosphoimager, and the amount of radioactivity in the inducible protein−DNA complex minus that present in the same position in the control (carrier solvent) sample lane represented the amount of inducible specific binding of the [^{32}P]DRE oligonucleotide. The amount of TA- or IAA-induced protein−DNA complex was expressed relative to that induced by TCDD.

Cell Culture, Chemical Treatment, and Luciferase Measurement. Recombinant mouse hepatoma cells (H1L1.1c2)

were grown and maintained as previously described (*43*). These cells were derived from Hepa1c1c7 cells and contain a stably integrated DRE-driven firefly luciferase reporter gene plasmid construct whose transcriptional activation occurs in a ligand- and AhR-dependent manner (*43*). H1L1.1c2 cells, grown in 96-well microplates, were incubated with carrier solvent [DMSO, ethanol, or water (20 μL/mL)], TCDD (2 nM), TRP (2 mM), or the indicated TRP metabolite (2 mM) for 4 h at 37 °C. After incubation, sample wells were washed twice with phosphate-buffered saline, followed by addition of cell lysis buffer (Promega), shaking of the plates for 10−20 min at room temperature to allow complete cell lysis, and measurement of luciferase activity in each well using a Dynatech ML3000 Microplate Luminometer with automatic injection of Promega stabilized luciferase reagent. Luciferase activity, normalized to the sample protein concentration using the fluorescamine protein assay (*44*) with bovine serum albumin as the standard, was expressed relative to that induced by TCDD.

Animal Treatment, Microsomal Preparation, and Measurement of Ethoxyresorufin O-Deethylase (EROD) Activity. βNF (30 mg/kg in corn oil) was injected intraperitoneally into Sprague-Dawley rats for 3 consecutive days. Twenty-four hours after the last treatment, the rats were sacrificed under ether anesthesia and the livers were removed following perfusion with ice-cold 0.9% NaCl. Livers were quick frozen in liquid nitrogen and stored at −80 °C until they were used. For preparation of microsomes, tissues were homogenized on ice in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose (3 mL/g of liver) using a Potter-Elvjheim homogenizer. Microsomes were prepared by centrifuging the liver homogenates at 9000*g* for 30 min, then centrifuging the resulting supernatant at 105000*g* for 90 min, and resuspending the 105000*g* pellet in 0.1 M Tris-HCl buffer (pH 7.5). The microsomal suspension was stored at −80 °C until it was used.

Ethoxyresorufin *O*-deethylase (EROD) activity was measured in hepatic microsomes according to the method of Rodrigues and Prough (*45*). The assay was adapted for use in 96-well plates and a fluorometric plate reader (Cytofluor 2350, Millipore). The reaction conditions (in a total volume of 200 μL) were 0.1 M Tris-HCl buffer (pH 7.8), 1 mg of BSA/mL, 0.1 mM NADPH, 0.6 μM ethoxyresorufin, and 100 μg of microsomal protein/mL. The reaction mixture was preincubated at 37 °C for 5 min without NADPH, and the reaction was started by the addition of NADPH. After 5 min, the reaction product resorufin was measured in the Cytofluor plate reader and the relative fluorescence compared to a calibration curve (0−150 nM resorufin). All incubations were carried out in duplicate and corrected for a background activity in the absence of NADPH and values expressed as fluorescent units of product formed per 5 min per milligram of protein.

RESULTS

TRP, TA, and IAA Induce AhR Transformation and DNA Binding in Vitro, While 5-OH-TRP, 5-OH-TA, MEL, and 5-MO-TA Did Not. We have previously demonstrated that the AhR can be transformed in vitro into its high-affinity DNA binding form by TCDD and other AhR ligands (*29*, *46*). The gel retardation analysis examining the ability of

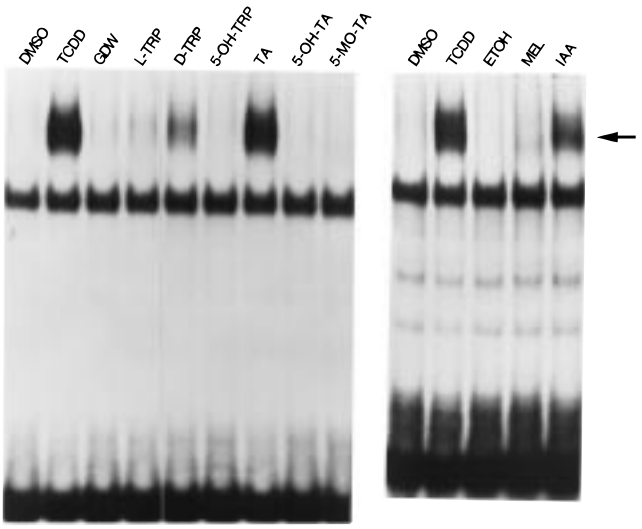

FIGURE 1: TRP and TRP metabolites stimulate AhR transformation and DNA binding in vitro. Guinea pig hepatic cytosol (16 mg/mL) was incubated with control solvents [DMSO, distilled water, or ethanol (20 mL/mL)], 20 nM TCDD (in DMSO), 2 mM L-TRP, D-TRP, 5-OH-TRP, TA, 5-OH-TA or 5-MO-TA (in water), or 2 mM MEL or IAA (in ethanol) for 2 h at 20 °C. Aliquots of each sample were mixed with [^{32}P]DRE and protein−DNA complexes resolved by gel retardation analysis as described in Materials and Methods. The arrow indicates the position of the inducible TCDD−AhR−DRE complex.

TRP or several TRP metabolites to induce AhR transformation and DNA binding of guinea pig hepatic AhR is shown in Figure 1. Incubation of cytosol with TA, IAA, and D-TRP and subsequent gel retardation analysis revealed that each compound induced formation of a protein−DNA complex which migrated to the same position as that produced by TCDD; small amounts of complex were observed with L-TRP and MEL, while no complex was observed with 5-OH-TRP, 5-OH-TA, and 5-MO-TA. This inducible complex has been previously demonstrated to represent the transformed AhR complex bound to the DRE (*11*, *12*, *41*, *42*, *46*). Comparable results were also observed using rat hepatic cytosolic AhR complexes (data not shown). Examination of the relative dose-dependent ability of TA and IAA, the most potent activators, to induce AhR transformation and DNA binding (Figure 2) revealed that TA was slightly more potent than IAA (EC_{50} of ∼0.2 and ∼0.5 mM, respectively), but both were significantly less potent than TCDD (EC_{50} ∼ 0.1 nM). These results demonstrate that the AhR can be transformed into its DNA binding form by several naturally occurring, relatively hydrophilic metabolites of TRP, albeit at relatively high concentrations.

TRP and TRP Metabolites Induce AhR Transformation, Nuclear Accumulation, and DNA Binding in Mouse Hepatoma Cells in Culture. To determine whether the chemicals which induced AhR DNA binding in vitro (TA, IAA, L-TRP, D-TRP, and MEL) could induce AhR transformation, nuclear accumulation, and DNA binding, nuclear extracts from mouse hepatoma (Hepa1c1c7) cells which had been incubated with each chemical for 1 h were analyzed by gel retardation (Figure 3). Consistent with the in vitro DNA binding results, L-TRP, D-TRP, TA, and IAA induced nuclear accumulation and DNA binding of the AhR complex; no complex formation was observed with MEL. Significantly more induced protein−DNA complex was observed with

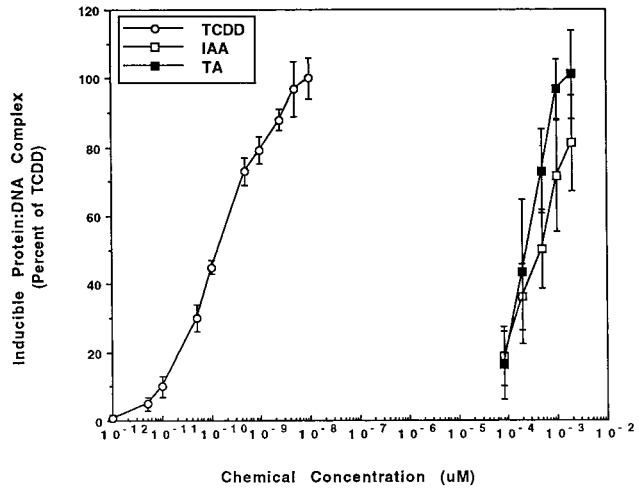

FIGURE 2: Dose-dependent activation of AhR DNA binding activity by TA and IAA. Guinea pig hepatic cytosol (16 mg/mL) was incubated with increasing concentrations of TCDD, TA, or IAA, and the formation of the protein−DNA complex was determined by gel retardation analysis and the amount of specific, chemical-induced protein−DNA complex formation determined by phosphorimaging analysis as described in Materials and Methods. The values are expressed as the mean ± standard deviation (SD) relative to the amount of TCDD-induced protein−DNA complex.

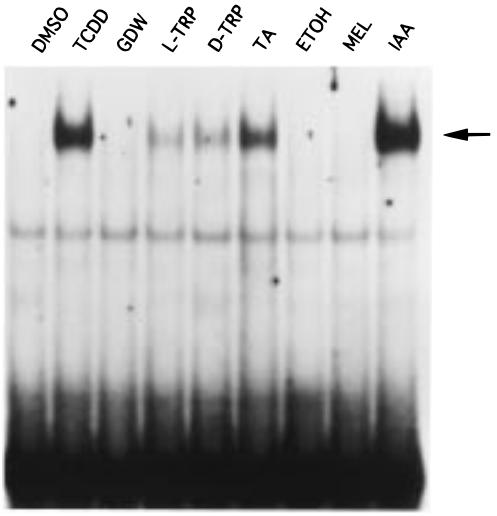

FIGURE 3: TRP and TRP metabolites stimulate nuclear accumulation of transformed AhR complexes in cells in culture. Nuclear extracts (6 μg of protein), prepared from mouse hepatoma (Hepa1c1c7) cells which had been treated for 1 h with DMSO (1 μL/mL), TCDD (1 nM), water (water) (1 μL/mL), 2 mM L-TRP, D-TRP or TA, ethanol (1 μL/mL), 2 mM MEL, or IAA, were incubated with the wild-type [^{32}P]DRE oligonucleotide and the amounts of protein−DNA complexes determined by gel retardation analysis. The arrow indicates the position of the induced protein−DNA complex.

nuclear extracts from TA- and IAA-treated cells compared to extracts from L-TRP- or D-TRP-treated cells. Further analysis of the TA- and IAA-induced nuclear protein DNA complexes revealed that the induced complexes were eliminated by a 50-fold excess of unlabeled wild-type DRE, but not by an excess of mutant DRE oligomer (Figure 4), which is comparable to the situation observed with nuclear extracts from TCDD-treated cells. These results confirm that the protein−DNA complex induced by TA or IAA exhibits the same DNA binding specificity as that of the TCDD−AhR complex and imply that the complex represents TA- and

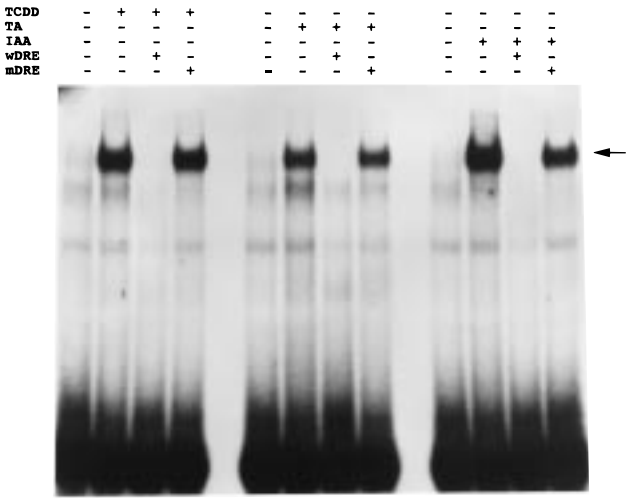

FIGURE 4: TRP and TRP metabolites stimulate nuclear accumulation of transformed AhR complexes in cells in culture. Nuclear extracts (6 μg of protein), prepared from hepa1c1c7 cells which had been treated for 1 h with DMSO (1 μL/mL), 1 nM TCDD, 2 mM TA, or 2 mM IAA, were incubated with the wild-type [^{32}P]-DRE oligonucleotide in the absence or presence of a 100-fold molar excess of unlabeled wild-type DRE or mutant DRE oligomer and the amounts of protein−DNA complexes determined by gel retardation analysis. The arrow indicates the position of the induced protein−DNA complex.

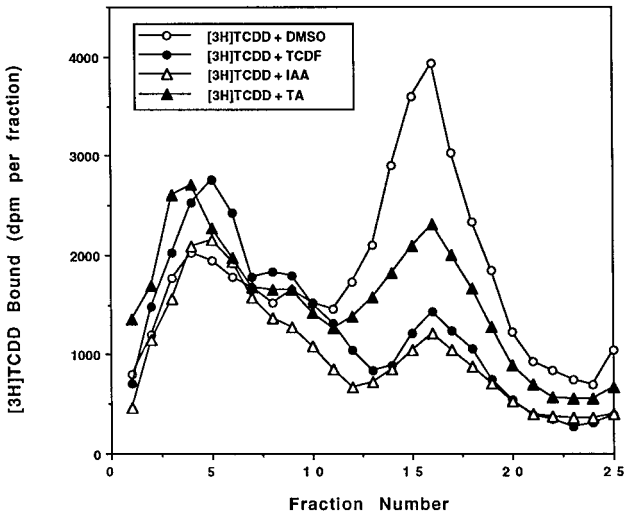

FIGURE 5: TA and IAA compete with [^{3}H]TCDD for binding to the cytosolic AhR. Guinea pig hepatic cytosol (5 mg of protein/mL) was incubated with 10 nM [^{3}H]TCDD in the absence (○) or presence (●) of 1 μM TCDF and in the presence of 2 mM TA (▲) or 2 mM IAA (△) for 1 h at 4 °C. Aliquots (300 μL) of each incubation were analyzed by sucrose density centrifugation as described in Materials and Methods.

IAA-transformed AhR complex bound to the DRE. Thus, the ability of TA and IAA to transform the AhR into its DNA binding form and to stimulate its nuclear accumulation suggests that these chemicals are AhR ligands.

TA and IAA Competitively Inhibit [^{3}H]TCDD Binding to the AhR. To directly demonstrate that AhR activation by TA and IAA is due to the direct binding of these chemicals to the AhR ligand binding site, we carried out competitive AhR binding analysis using [^{3}H]TCDD (Figure 5). Sucrose gradient analysis using guinea pig hepatic cytosol revealed that TA and IAA could competitively inhibit [^{3}H]TCDD binding to the AhR, with IAA being a more effective competitor; no significant decrease in [^{3}H]TCDD was

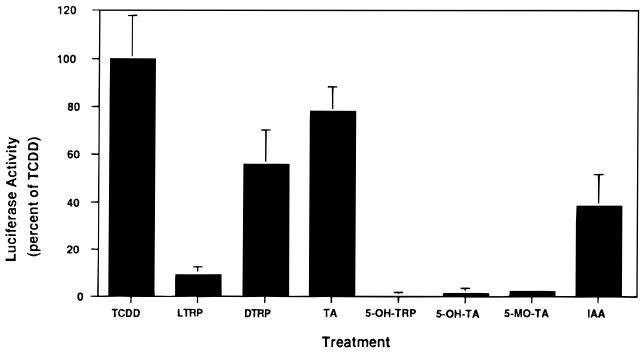

FIGURE 6: Induction of luciferase reporter gene activity in stably transfected mouse hepatoma (H1L1.1c2) cells by TRP and TRP metabolites. Confluent 96-well plates of cells were incubated with control solvents (DMSO, water, or ethanol at 2.5 μL/125 μL), 1 nM TCDD or 2 mM TRP, or the indicated TRP metabolite for 4 h at 37 °C. Luciferase activity in cell lysates was determined as described in Materials and Methods. Values represent the mean ± SD of at least triplicate determinations and were expressed relative to the activity obtained with TCDD.

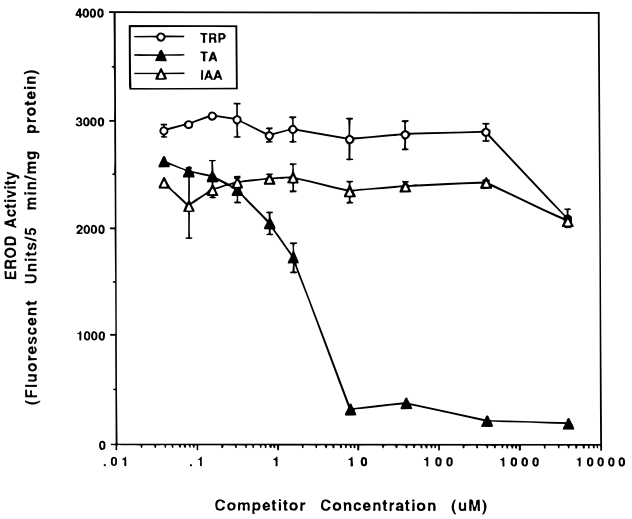

FIGURE 7: TA, but not L-TRP or IAA, is an inhibitor of CYP1A1-dependent EROD activity. Rat hepatic microsomal preparations were incubated with increasing concentrations of L-TRP, TA, or IAA, and EROD activity was determined as described in Materials and Methods. Values are expressed as the mean ± SD of triplicate determinations with the fluorescent units of product produced per minute per milligram of protein.

observed with L-TRP or D-TRP (data not shown). The high concentration of TA and IAA needed to competitively displace [^{3}H]TCDD indicates that these chemicals are relatively weak AhR ligands, compared to TCDD.

TA and IAA Induce AhR-Dependent Gene Expression. Although TRP, TA, and IAA can bind and transform the AhR into its DNA binding form in vitro and in Hepa1c1c7 cells, it remains to be determined whether they are able to induce AhR-dependent gene expression. To examine this aspect, we utilized a recently developed recombinant mouse hepatoma cell line (H1L1.1c2) which contains a stably integrated firefly luciferase gene whose expression is under AhR-dependent control of four DREs (*43*). We have previously demonstrated that treatment of these cells with AhR ligands (agonists) induces luciferase gene expression in a time-, dose-, and AhR-dependent manner (*43*). The results of treatment of the H1L1.1c2 cells for 4 h with various TRP and TRP metabolites are shown in Figure 6. The results of these experiments are relatively consistent with the DNA binding studies in that they revealed that TA and IAA induced significant levels of reporter gene expression, and the 5-hydroxylated TRP metabolites were virtually inactive. Interestingly, D-TRP was consistently a significantly better inducer of gene expression than would be predicted on the basis of its in vitro ligand and DNA binding activity.

TA Is a Substrate for Cytochrome P4501A1-Dependent Monooxygenase Activity. Although TA and IAA can induce AhR-dependent gene expression, the physiological relevance of this induction is unknown. Given the fact that CYP1A1 transcription is induced following exposure to AhR ligands, combined with its documented ability to metabolize many AhR ligands and other structurally diverse chemicals (*47*), it is possible that TRP, TA, or IAA could act as a substrate for P4501A1. To assess this, we examined the ability of each compound to competitively inhibit EROD, P4501A1-dependent monooxygenase activity (*4*, *45*). A concentration-dependent inhibition of EROD activity was observed with TA but not with IAA or L-TRP (Figure 7). The inhibition by TA appeared to be of a competitive nature because the addition of more ethoxyresorufin to the incubation mixture reversed much of the TA-dependent inhibition of EROD activity (data not shown). In addition, TA also appears to be a relatively good competitive substrate for P4501A1 since its IC_{50} for EROD inhibition was 4 mM, about 7-fold greater than the 0.6 mM concentration of EROD substrate ethoxyresorufin present in the incubation.

DISCUSSION

Recent studies have demonstrated that several indole-containing chemicals are, or can be converted into, AhR ligands. For example, 7,8-dihydrorutacarpine and indole-3-carbinol are two naturally occurring indole-containing chemicals found in plants which can bind to and activate AhR (*14*, *17*, *18*). In the mammalian digestive tract, metabolic conversion of dietary indoles (including indole-3-carbinol and TRP) to significantly more potent AhR ligands (*17*, *27*), as well as conversion of TRP by UV light into several photoproducts which are high-affinity AhR agonists, has also been described (*28*, *29*). Here we demonstrate that endogenous indole-containing compounds such as TA and IAA, and to a lesser degree L- and D-TRP, are ligands for AhR, and although these chemicals are relatively weak ligands compared to TCDD, they exhibit full AhR agonist activity (i.e., they bind to the AhR, induce AhR nuclear transport, and activate gene expression in an AhR- and DRE-dependent manner). While a comparison of L-TRP, D-TRP, TA, and IAA with respect to relative binding and AhR nuclear accumulation revealed that IAA and TA were the most potent agonists, D-TRP was more active than IAA with regard to promoter activity in mouse hepatoma cells. The reason for the greater potency of D-TRP compared to L-TRP is unknown, but may result from the metabolic conversion of D-TRP into another more potent ligand and/or differences in its uptake by the cell.

Miller (*35*) has recently reported that relatively high concentrations of TRP, indole, IAA, and TA induced transcription of the CYP1A1 promoter in an AhR-dependent manner in a recombinant yeast system. However, this study did not directly demonstrate that similar responses also occur

in mammalian cells. This is an important issue especially given that the ligand binding specificity of the AhR expressed in yeast appears to differ somewhat from that in mammalian cells (*36*, *37*). Our results not only confirm the findings of Miller (*32*) but also demonstrate that these chemicals (and/or their metabolites) can not only transform AhR into its DNA binding form but also induce AhR-dependent gene expression in cultured mouse liver cells. Our ligand binding results are in apparent contrast to those of Gillner et al. (*48*), who reported little binding of TRP, IAA, and TA to rat hepatic cytosolic AhR. The inability of Gillner et al. (*48*) to demonstrate competitive AhR binding by these chemicals is likely due to the fact that the concentrations of these weak ligands in his assay were too low to allow them to effectively compete with [^{3}H]TCDD, an extremely high-affinity ligand. This substantial difference in potency is evidenced in the gel retardation results (Figure 2), where TCDD is $\sim 10^6$-fold more potent than TA or IAA.

Our results, as well as those of Miller (*35*), indicate that TRP (and TRP metabolites) can activate the AhR signaling pathway. However, given the relatively high concentration of these chemicals necessary for the induction and AhR activation, it is possible that a contaminant(s) and/or an oxidative metabolite with extremely high potency and/or affinity present in these preparations may be responsible, at least in part, for the observed AhR-dependent activity. To address these concerns, a solution of TA (our most active chemical) was fractionated by reverse phase HPLC and the ability of each fraction to activate AhR was determined. Although this separation protocol revealed that the TA preparation contained several minor contaminants, the AhR activation activity was found to be associated only with the main TA peak (data not shown). These results suggest that the parent compound, and/or a contaminant that coeluted with TA, is responsible for the AhR-dependent activity. Miller (*35*) similarly concluded that his observed TRP-dependent induction of gene expression was unlikely to be due to a contaminant in the TRP preparation. The lack of AhR-dependent activity of other TRP metabolites (which are synthesized and purified in a similar manner) and the consistent AhR-dependent activity of TRP and TA obtained from different commercial sources (data not shown) also suggest that the TRP metabolites are the actual AhR-activating species. Finally, the fact that TA dissolved in water can activate the AhR would argue against the presence of any known high-affinity AhR agonist in this preparation because these latter chemicals are generally extremely hydrophobic and have very limited water solubility. However, we cannot conclusively rule out the possibility that there may be a high-affinity ligand with these characteristics in this preparation.

The above results not only identify TA as an AhR agonist and as a competitive substrate for cytochrome P4501A1 but also provide some physiological relevance to our results by suggesting that TA and possibly other indole-containing chemicals can stimulate their own metabolism, a feature common to many AhR ligands (*49*). This seems more significant when one considers the fact that many TRP metabolites are neuroactive, including quinolinic acid, kyurenine, *N*-acetyltryptophan, tryptophol, IAA, 5-OH-TA, MEL, and *N*,*N*-dimethyltryptamine (*31*−*34*). The association of an altered TRP metabolism with Parkinson′s disease and schizophrenia and the identification of high-affinity TA receptors which appear to be involved in normal neurological and psychiatric functions (*50*, *51*) suggest that alterations in TRP metabolism could be a factor in the etiology of several psychiatric disorders (*52*). Neurobehavioral and neurochemical effects of TCDD and coplanar PCBs on the central nervous system have been reported, and the results are still controversial. Although TCDD does not penetrate the brain very well and levels are far lower than those seen in the liver and adipose tissue (*53*), sensitive biochemical effects, such as enzyme induction, have been observed in brain tissue from TCDD-exposed rats (*54*). In addition, subtle behavioral differences, such as learning impairment and more aggressive behavior patterns, have been observed in TCDD-treated monkeys (*55*, *56*) and suggestions of developmental or psychomotor delays in children exposed to polychlorinated biphenyls and dibenzofurans in utero (*57*, *58*). There is also in vivo evidence of enhanced TA metabolism in brains of offspring born to PCB-exposed dams (*59*), and there are reports that TCDD treatment decreases brain levels of TRP and 5-OH-TA in rats (*60*). Given that AhR (*61*) and ARNT (*62*) have been detected in rats brains using in situ hybridization techniques and the induction of brain CYP1A1 gene expression by HAHs and PAHs in rats (*63*), humans (*64*), and trout (*65*) has been demonstrated, it is possible that potentially serious physiological and toxicological consequences could occur as a result of a perturbation of TRP metabolism by AhR ligands, particularly in the brain.

The ability of TCDD to induce an anorexia-like wasting syndrome in a wide variety of animals is well-documented (*4*, *5*). Although the exact mechanism(s) by which this wasting response occurs is unknown, one characteristic of anorexia is a rise in free TRP levels and the subsequent increase in TRP transport across the blood−brain barrier. In humans, it has been shown that an increase of free TRP causes a greater increase in the levels of metabolites of TA, such as IAA, as opposed to 5-OH-TA (*66*). Similarly, Unkila and co-workers not only reported that rats exposed to TCDD exhibited a significant increase in plasma-free TRP but also noted a strong association between altered brain TRP metabolism and feeding behavior (*60*). It should be pointed out, however, that although the effects of TCDD on wasting and lethality are correlated with increased 5-OH-TA turnover in rats (*60*), similar effects were not observed in the most TCDD susceptible species, guinea pigs (*67*). Thus, additional work is needed to determine the role (if any) of TRP metabolism and TRP metabolites in TCDD-induced wasting syndrome.

The ability of TRP and TRP metabolites to act as AhR ligands is also potentially significant from another point of view. Analysis of the structure of the AhR and ARNT proteins reveals that each contains a highly conserved structural domain called PAS [for the region of structural homology between the proteins PER, ARNT, AhR, and SIM (*1*−*3*)]. In the AhR, the PAS domain not only contains the AhR ligand and hsp90 binding domains but also plays a critical role in the dimerization of the PAS family members (*1*−*3*). Recently, the cloning of the cDNAs for numerous other PAS domain proteins has been reported; not only do the majority of these proteins appear to be transcription factors, but several of these PAS family members appear to be involved in the processes of circadian rhythm. Examples

include the *Drosophila* protein PER, a periodicity gene important in circadian rhythm in this species (*68*), and the two recently cloned genes encoding murine PAS-containing circadian clock proteins (*69*, *70*). An intriguing tie between this information and our data is the relationship between the modulation of circadian rhythm by TRP and the altered timing of feeding behavior (*71*). Since it is possible that other members of the PAS family of proteins may also be ligand-dependent, the potential regulation of some of these proteins by indole-containing ligands is an interesting direction for future studies.

Physiological TRP, TA,and IAA levels that have been reported for humans vary widely among tissue types. Plasma TRP levels are reported to be in the range of 70 (*71*, *72*) to 150 μM (*67*, *72*), while plasma IAA levels appear to be substantially lower, ~2.8 nM (*73*). In contrast, accurate measurement of tissue TA levels has been difficult since broken cell extracts contain enzymes, in particular monoamine oxygenases (MAOs), which rapidly convert TA to IAA; therefore, most reported values for TA are very low and probably do not reflect physiological levels. However, in the presence of MAO inhibitors, tissue concentrations of TA have been reported to be as high as 120 ng/g (~700 μM) (*50*). Thus, although TRP, TA, and IAA can activate AhR and induce AhR-dependent gene expression in vitro and in mouse hepatoma cells in culture, it is unlikely that they influence AhR-dependent gene expression to any degree under "normal" physiological conditions in vivo. However, whether appropriate physiological or pathological conditions might occur in vivo to allow TRP or a TRP metabolite to activate AhR in specific cell types or tissues remains to be determined.

Overall, we have used several AhR-dependent bioassay systems to demonstrate that endogenous indole-containing compounds (such as TA, IAA, and TRP) are water-soluble ligands and/or activators for AhR. Given the promiscuous nature of AhR ligand binding (*14*), it is likely that multiple endogenous activators of the AhR exist. In ongoing studies in our laboratory, we are using analogous experimental approaches to identify and characterize other natural and endogenous AhR ligands, and to elucidate the role of TRP and/or its metabolites in dioxin-induced toxicity.

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