Use of [^{125}I]4′-Iodoflavone as a Tool to Characterize Ligand-Dependent Differences in Ah Receptor Behavior

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ABSTRACT: We have synthesized [^{125}I]4′-iodoflavone to study Ah receptor (AhR)–ligand interactions by a class of AhR ligands distinct from the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This radioligand allows the comparison of AhR–ligand interactions using a ligand that differs in AhR affinity, and yet has the same radiospecific activity as [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin. Specific binding of [^{125}I]4′-iodoflavone with the AhR was detected as a single radioactive peak (∼9.7 S) following density sucrose gradient analysis. Cytosolic extracts from both Hepa 1 and HeLa cells were used as the source of mouse and human AhR, respectively. A ∼6.7 S form of radioligand-bound Ah receptor was detected in the high salt nuclear extracts of both cell lines. In HeLa cells approximately twofold more [^{125}I]4′-iodoflavone–AhR 6 S complex, compared with [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin, was recovered in nuclear extracts. A comparison of the ability of 4′-iodoflavone and TCDD to cause time-dependent translocation of AhR-yellow fluorescent protein revealed that 4′-iodoflavone was more efficient at enhancing nuclear accumulation of the receptor. These results suggest that [^{125}I]4′-iodoflavone is a particularly useful and easily synthesized ligand for studying the AhR.
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KEYWORDS: **Aryl Hydrocarbon Receptor; TCDD; Dioxin; 4′-Iodoflavone**

INTRODUCTION

Polycyclic aromatic hydrocarbons and polychlorinated dibenzo-*p*-dioxins are widely dispersed throughout the environment and are a public concern because of their potential roles in chemically induced carcinogenesis. Environmental contamination with polychlorinated dioxins, for example 2,3,7,8-tetrachloro-*p*-dioxin (TCDD), has been shown to result from incinerator emissions [1] as well as from chemical dump leachates, such as that found at Love Canal [2]. Some evidence of polychlorinated dibenzo-*p*-dioxin contamination of indoor air due to wood preservation treatment [3] and cigarette smoke [4] has also been established, thus increasing possibilities for exposure to these compounds.

TCDD binds with high affinity to the Ah receptor (AhR) and mediates most, if not all, of the toxic effects of this compound [5,6]. Upon ligand binding, the AhR complex translocates into the nucleus where the 90-kDa heat shock protein dimer (hsp90) and the co-chaperone XAP2 dissociate and subsequently heterodimerize with Ah receptor nuclear translocator protein (ARNT). The AhR/ARNT heterodimer binds to dioxin response elements (DRE) leading to an alteration in the transcription rate of genes encoding proteins such as cytochrome P4501AI (See Refs. [7] and [8] for review). This induction is often detected by an increase in aryl hydrocarbon hydroxylase (AHH) activity.

Historically, most studies have focused on rodent AhRs, as the human AhR has been difficult to characterize. This is due, in part, to the human AhR's apparent lower affinity for ligands such as TCDD, and its low concentration and stability in both human tissues and cell lines, as compared to the rodent AhR in rodent cell lines [9,10]. Thus, limited information is available on AhR ligand interactions and

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how these interactions in the human receptor complex compare to those demonstrated with the rodent AhR. In addition, studies are needed to examine how different AhR ligands may induce different conformations in the AhR that may lead to significant changes in its biological activity (e.g. coregulator recruitment). AhR agonists other than TCDD include compounds such as 3-methylcholanthrene and 5,6-benzoflavone which characteristically induce AHH activity, but have relatively lower AhR affinity [11,12]. Although 4′-substituted flavones have previously been characterized as potent inducers of AHH activity [13,14], these compounds have been only partially characterized as AhR ligands [15]. In this study, we have synthesized [^{125}I]4′-iodoflavone ([^{125}I]FL), which represents a different class of polycyclic AhR ligands compared with dioxin analogues. [^{125}I]FL can be directly compared with [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin ([^{125}I]Br_2DpD) to characterize ligand-specific differences in the behavior of the AhR in cultured cells due to each compound having the same radiospecific activity. These studies indicate that [^{125}I]FL is a suitable ligand to study the behavior of the AhR.

MATERIALS AND METHODS

Chemicals

[^{125}I]Br_2DpD was synthesized as described previously [16]. α-Napthoflavone (αNF) and β-napthoflavone (βNF) were purchased from Aldrich Chemical Company (Milwaukee, WI). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) were obtained from Stephan Safe (Texas A & M University, College Station, TX). All other chemicals were obtained from Sigma (St. Louis, MO) unless noted elsewhere.

RNA Blot Analysis

Hepa 1c1c7 cells were treated with either solvent control (dimethyl sulfoxide) or the indicated chemicals for 14 h. Total RNA was isolated as previously described [17]. Ten micrograms of each sample was loaded onto a 1.2% agarose–formaldehyde gel and the RNA transferred to a nitrocellulose membrane following electrophoresis. The probe used for hybridization was the Pst1 insert of the pmP1450 plasmid (ATCC) that was ^{32}P-labeled using the random-prime method [18]. Following hybridization, the blot was washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 45°C for 1 h and twice with 0.3 × SSC–0.1% SDS for 1 h. A glyceraldehyde-3-phosphate dehydrogenase probe served as a loading control.

Synthesis of the Pyrrolidyl Derivative of 4′-Aminoflavone

The method has been adapted from that given by Foster et al. [19]. To a suspension of 4′-aminoflavone (1.0 g, 4.2 mmol) in water (10 mL) at 0°C was added 5 mL of ice-cold aqueous solution of sodium nitrite (320 mg, 4.6 mmol). The mixture was stirred on ice while trifluoroacetic acid (970 μL, 12.6 mmol) was added dropwise. After stirring for 5 min, the solution was added dropwise to ice-cold pyrrolidine (420 μL, 5.0 mmol) in 1.1 M KOH (10 mL). The reaction was stirred for 10 min on ice, adjusted to pH 7 with 1.1 M KOH, and the crude solid product filtered. The solid was washed with water followed by methanol, to give 1.2 g (89% yield) of the pyrrolidyl triazine. Purification by flash chromatography (silica, $CHCl_3$/EtOAc, 20:1) followed by recrystallization from ethanol yielded fine yellow needles, m.p. 178–179°C.

Synthesis of 4′-Iodoflavone

To a stirred solution of the above pyrrolidyl triazine (60 mg, 0.19 mmol) in methanol (25 mL) was added sodium iodide (58 mg, 0.38 mmol) in water (0.5 mL). Trifluoroacetic acid (60 μL, 0.78 mmol) was added dropwise and the clear orange solution stirred at 40°C for 7 h. The solvent was evaporated and the solid residue was washed with methanol and filtered to give 20 mg of crude product. Flash chromatography (silica, $CHCl_3$/EtOAc, 20:1) followed by recrystallization from methanol gave 4′-iodoflavone as white needles, m.p. 168°C (lit. 168–169°C, [13]). Analysis calculated for $C_{15}H_9IO_2$: C, 51.75; H, 2.61; I, 36.45. Experimentally determined: C, 51.64; H, 2.23; I, 36.18.

Synthesis of [^{125}I]FL

4′-Aminoflavone (46.5 mg) was dissolved in 1 mL methanol, and 10 μL of this solution was removed and dried. Ten microliters of dilute HCl (2% final concentration) was added to the dried 4′-aminoflavone, the solution was vortexed, and 10 μL of H_2O was added. The solution was cooled on ice, and sodium nitrite (0.149 mg in 10 μL H_2O, 2.16 μmol) was added while stirring and the mixture stirred on ice for 10 min. Six microliters of this solution was added to 5 mCi of NaI^{125} (NEN Research Products Boston, MA). The reaction was allowed to proceed for 2 h on ice. The reaction was stopped by the addition of 10 μL of 0.5 M NaOH and 25 μL of $Na_2S_2O_5$ (20 mg/mL). The product was extracted into 250 μL CH_2Cl_2 and the organic extract was dried down using the charcoal trapping system. The product was then solubilized in 20 μL of 80% methanol and the sample applied to a Beckman ultrasphere C18 column

(4.6 × 250 mm, 5-μm particle) in an isocractic solvent system (methanol/water, 80:20) at a flow rate of 0.5 mL/min. The compound was detected by its UV absorbance at 254 nm and eluted with a retention time of 21 min.

Cell Culture

Hepa 1c1c7 (Hepa 1) and c4/1 cells were obtained from Dr. James P. Whitlock (Dept. of Pharmacology, Stanford, CA) and c4/2 cells from Dr. Oliver Hankinson (Dept. of Pathology, Los Angeles, CA) and were maintained in (α-minimal essential medium (Sigma, St. Louis, MO) containing 8% fetal calf serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in 94% air/6% CO_2 mixture. HeLa cells were obtained from Dr. Jeffrey Ross (McArdle Laboratory for Cancer Research, University of Wisconsin-Madison) and were grown in (α-minimal essential medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. COS 1 cells were obtained from ATCC (Manassas, VA) and grown in the same medium as HeLa cells.

EROD Activity Assay

Trypsinized Hepa 1 cells were suspended in 0.25 M sucrose–0.05 M Tris (pH 7.5) and homogenized with a tight-fitting stainless steel Dura-Grind Dounce tissue grinder (Wheaton Instruments, Millville, NJ). Microsomes were isolated and utilized in EROD assays essentially as described [20].

Cytosolic Preparation

Hepa 1, c4/1, c4/2, or HeLa cells were harvested using trypsin/EDTA and washed in Dulbecco's phosphate-buffered saline. The cells were suspended in 25 mM MOPS, 1 mM EDTA, 0.02% NaN3 (pH 7.5) at 0°C with 10% glycerol (MENG) and homogenized in a Dura-Grind Dounce tissue grinder with 15 strokes by hand. Sodium molybdate (20 mM) was added to the buffer during cytosolic preparation of HeLa cell cytosol. Cytosolic and nuclear extracts were used immediately.

Ligand Binding Studies

Dilute cytosolic fractions from HeLa, c4, or Hepa 1 cells were incubated with 0.10 nM [^{125}I]radioligand for 1.5 h at 4°C. Excess ligand was removed by the addition of the protein/ligand mixture to a charcoal dextran pellet, at optimal concentrations of 0.24 mg charcoal:1 mg protein. But when the mixture included HeLa cytosol, the optimal charcoal concentration of the mixture was 0.14 mg charcoal/mg protein. The protein/ligand/charcoal mixture was incubated for 15 min at 4°C and the charcoal was removed by centrifugation (3000 × g) for 10 min at 4°C. A 300-μL aliquot of the supernatant was removed and either counted for analysis of specific binding or applied to sucrose gradients.

Binding of 4′-iodoflavone, αNF, βNF, and TCDF to the AhR was measured by each compound's ability to compete with [^{125}I]Br_2DpD for specific binding sites. This was performed by adding increasing concentrations of competing ligand in DMSO to cytosol (150 μg/mL) containing 0.26 nM [^{125}I]Br_2DpD. ([^{125}I]Br_2DpD at a concentration of 0.26 nM, was determined to fully saturate all AhR binding sites at the protein concentration used (data not shown).) The protein/ligand mixture was incubated for 30 min at 25°C. Excess ligand was removed following the addition of dextran/charcoal and subjected to centrifugation as described above [20]. Nonspecific binding was defined as the amount of radioligand bound in the presence of a 200-fold molar excess of TCDF. Specific binding was determined by subtracting nonspecifically bound radioligand from total radioligand bound. IC_{50} and K_i values were calculated as described previously using a Logit Y vs. Log_{10}[competitor]/[^{125}IBr_2DpD] plot [21,22]. Values represent the means ±SD obtained from three separate experiments. Correlation coefficients were greater than 0.98. The K_D value of the AhR was approximated by the method of Scatchard using [^{125}I]Br_2DpD as the radioligand [23].

Sucrose Density Centrifugation

The sample was applied to 10–30% sucrose gradients (5.1 mL) containing the same buffer as the samples analyzed. The tubes were centrifuged for 135 min at 385,000 × g and 200 μL fractions were collected using a model 640 density gradient fractionator (ISCO, Inc., Lincoln, NE). The radioactivity of each sample was counted in a model 1191 gamma counter (Tm Analytic, Inc., Elk Grove Village, IL). Bovine serum albumin (4.4 S) and catalase (11.3 S) were used as external standards for approximating molecular size.

Nuclear Extract Isolation

HeLa and Hepa 1 cells were grown to near confluency. Either [^{125}I]FL or [^{125}I]Br_2DpD (specific activity, 2176 Ci/mmol, in DMSO) was added to the medium to give a final concentration of 0.52 nM and allowed to incubate at 37°C for 1 h. Additional flasks were incubated with either [^{125}I]FL and a 20-fold molar excess of TCDF or [^{125}I]Br_2DpD and a 200-fold excess of TCDF. The cells

were harvested and homogenized as described above. The cell homogenates were centrifuged at 900 × *g* for 15 min, the supernatant removed, and the nuclear pellet washed three times with MENG. The pellet was resuspended and incubated in 300 μL of MENG (including 20 mM sodium molybdate with HeLa cells) and 400 mM potassium chloride for 1 h at 4°C. The nuclear fraction was recovered following centrifugation at 100,000 × *g* for 1 h and applied to sucrose density gradients.

Gel Shift Analysis

Gel shift analysis was performed using Hepa 1 nuclear extracts essentially as described [15].

Fluorescence Microscopy

COS 1 cells growing in 6-well microplates were transfected with 0.5 μg pEYFP-mAhR and 1.0 μg pCI-XAP2 using LipofectAMINE with PLUS reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instruction's. The next day following transfection (~18 h), cells were visualized by fluorescence microscopy with a Nikon TE300 inverted microscope with TE FM epi-fluorescence attachment using a Nikon Pan Fluor 60X objective and a SPOT RT Color model 2.2.0 cooled CCD camera. Cells were treated with either 10 nM TCDD or 10 mM iodoflavone in DMSO and observed for a total of 6 h. Each observation consisted of the scoring of the localization of the mAhR-YFP in approximately 150 cells. Localization was scored into five categories: cytoplasmic, cytoplasmic > nuclear, cytoplasmic = nuclear, nuclear > cytoplasmic, and nuclear. For the plots, cytoplasmic = nuclear was discarded, cytoplasmic and cytoplasmic > nuclear were combined to give C > N, and nuclear > cytoplasmic and nuclear were combined to give N > C.

RESULTS

Limited information is available describing ligand-dependent differences in AhR activation and subsequent transcriptional activity. A number of antagonists and agonists have been shown to result in inhibition or stimulation of AhR activity, respectively [12,24]. However, the ability to study the biochemical properties of the liganded AhR is limited by the lack of suitable radioligands. We decided to search for AhR ligands that could be easily produced with high radiospecific activity and could complement the useful dioxin analogue [^{125}I]Br_2DpD, which is already available [16]. Another criteria that had to be met was that the ligand must have intermediate affinity, but still be capable of binding with sufficient affinity to allow radioligand binding studies to be performed. An additional requirement was to find a compound that would represent a class of AhR ligands distinct from the chlorinated dibenzofurans or dibenzodioxins. One compound that appeared to meet all of these criteria is 4′-iodoflavone.

Characterization of 4′-Iodoflavone as an AhR Agonist

As shown in Table 1, 4′-iodoflavone displays approximately the same affinity for the AhR as βNF. The IC_{50} and K_i values of different ligands reflect the ability of the compounds to compete with [^{125}I]Br_2DpD for AhR binding. Table 1 reveals that 4′-iodoflavone has a relative affinity that is almost 40-fold lower than TCDF and thus 4′-iodoflavone could be described as a ligand with intermediate affinity. However, the AhR's affinity for αNF was approximately 10-fold lower than for 4′-iodoflavone. The relative affinities determined in this study are in excellent agreement with those obtained previously [11,23]. For example, the K_i values obtained for βNF and αNF were calculated to be 1.5 and 15 nM, respectively, using competition assays with [^{3}H]TCDD–AhR specific binding in rat liver cytosol [25]. The K_D value for [^{125}I]Br_2DpD determined in this study, 43.5 pM at 150 μg/mL, is comparable to the values of 160 pM and 12 pM calculated using cytosolic protein concentrations of 1160 μg/mL and 36 μg/mL, respectively. Conditions were similar to those used in the present study, including the use of [^{125}I]Br_2DpD as the radioligand, although C57BL/6J mouse liver was used as the protein source [16]. If 4′-iodoflavone is an agonist for the AhR it should be capable of inducing EROD activity in cells. Hepa 1 cells were treated with TCDD or 4′-iodoflavone at several different concentrations, cells were lysed, and EROD assays performed. The data in Table 2 indicates that 4′-iodoflavone is capable of inducing EROD activity at relatively low concentrations. However, at higher concentrations 4′-iodoflavone actually inhibited EROD activity, in contrast to results obtained with TCDD, an effect that has been

TABLE 1. Comparison of IC_{50} and K_i Values of Competing Ligands[a]

Competitor	IC_{50}[b] *(nM)*	K_i[b] *(nM)*
2,3,7,8-Tetrachlorodibenzofuran	0.59 ± 0.1	0.09 ± 0.002
β-Naphthoflavone	8.45 ± 0.4	1.64 ± 0.300
4′-Iodoflavone	12.2 ± 0.2	3.89 ± 0.67
α-Napthoflavone	131 ± 3.3	29.0 ± 0.62

[a] Values represent the mean ± SD from three separate experiments. The K_D value for the AhR, using [^{125}I]Br_2DpD as the radioligand and Hepa 1 cytosolic protein (150 μg/mL), was determined to be 43.5 pM using Scatchard analysis.
[b] IC_{50} and K_i values were calculated as described in Materials and Methods.

TABLE 2. Induction of EROD by Ah Receptor in Hepa 1c1c7 Cells

Ligand	*Concentration (M)*	*EROD (pmol/mg protein/min)*
DMSO (control)	–	1.01 ± 0.07
IFL	2.5^{-7}	13.17 ± 2.01
IFL	5.0^{-7}	8.41 ± 0.92
IFL	5.0^{-6}	3.46 ± 0.83
IFL	1.0^{-5}	3.91 ± 1.42
TCDD	5.0^{-11}	71.72 ± 7.73
Plus IFL	2.5^{-7}	33.75 ± 5.41
Plus IFL	5.0^{-7}	19.03 ± 1.42
Plus IFL	5.0^{-6}	8.35 ± 1.94
Plus IFL	1.0^{-5}	7.04 ± 1.11
Plus βNF	1.0^{-5}	58.32 ± 8.25
Plus αNF	1.0^{-5}	18.18 ± 0.11

TABLE 3. Effect of Iodoflavone on DNA Binding in Hepa 1c1c7 Cells

Treatment	*Net (cpm)*	*% of Maximum Response*
TCDD (10^{-9} M)	3210 ± 583	100
Iodoflavone (10^{-6} M)	6147 ± 1278[a]	184 ± 30[a]
Iodoflavone (10^{-7} M)	3453 ± 723	93 ± 14
Iodoflavone (10^{-8} M)	2977 ± 1016	103 ± 37
TCDD (10^{-9} M) + Iodoflavone (10^{-6} M)	3545 ± 1317	110 ± 33

Results show means of triplicate determinations from one nuclear extract preparation. The significance of co-treatment with Iodoflavone on DRE binding compared to treatment with TCDD treatment alone was assessed by Student's *t* test.

[a] $p < 0.01$.

previously described [15]. Treatment with both iodoflavone and TCDD also resulted in a dose-dependent decrease in EROD activity. This data would suggest that 4′-iodoflavone is probably a substrate or inhibitor for cytochrome P450 1A1 that competes with substrate provided in the EROD assay. Northern blot analysis was performed to assess whether 4′-iodoflavone is capable of inducing CYP1A1 mRNA synthesis. Results indicated that 4′-iodoflavone was capable of increasing mRNA levels, although not to the same extent as TCDD (Figure 1). This would indicate that 4′-iodoflavone is an agonist for the AhR. To further establish that 4′-iodoflavone is indeed an AhR agonist, gel shift analysis was performed. The results clearly indicate that 4′-iodoflavone is highly efficient in transforming the AhR to the AhR/ARNT heterodimer, which then leads to binding to a DRE oligonucleotide (Table 3). Interestingly, at high ligand concentrations 4′-iodoflavone was capable of forming approximately twofold more shifted AhR/ARNT complexes than TCDD. Also, the addition of both TCDD and 4′-iodoflavone leads to a level of DNA complex seen with TCDD alone. This would suggest that 4′-iodoflavone at high concentrations may be more efficient than TCDD in mediating AhR/ARNT complex formation and subsequent binding to DNA. It is also important to consider that 4′-iodoflavone should have greater solubility in cell culture medium as well as within the cell compared with TCDD.

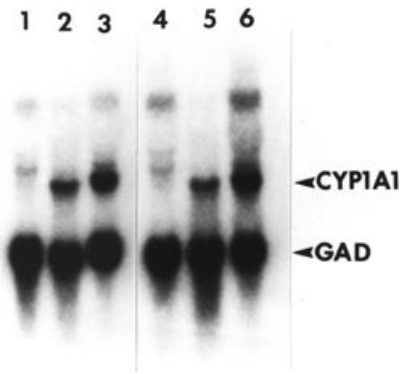

FIGURE 1. RNA blot analysis of CYP1A1 mRNA induction by TCDD and iodoflavone. Hepa 1 cells were cultured for 14 h in the presence of AhR ligands and total RNA was isolated. RNA was separated on an agarose gel transferred to nitrocellulose and the membrane probed with both a radiolabeled CYP1A1 and a glyceraldehyde-3-phosphate dehydrogenase (GAD) probe as a control. The following samples from treated Hepa 1 cells were examined: Lanes 1 and 4, control solvent, Lanes 2 and 5, 10 μM iodoflavone, Lanes 3 and 6, 10 nM TCDD. The high molecular weight band observed in Lanes 1, 3, 4, and 6 are nonspecific binding of the probes to 20 S rRNA.

Synthesis of Iodoflavone

Iodination of aryl amines has been shown to proceed from stable pyrrolidyl triazine intermediates [26]. The pyrrolidyl triazine derivative of 4′-aminoflavone was isolated and purified, from which a facile synthesis of 4′-iodoflavone was achieved. Pure, unlabeled 4′-iodoflavone was thus available as an HPLC standard. However, subjecting the pyrrolidyl triazine intermediate to the reaction conditions approximating those of a radiosynthesis (i.e., low quantities of NaI in aqueous NaOH) gave unacceptable product yields. For the synthesis of [^{125}I]FL the classic diazotization method was therefore employed. The synthesis of [^{125}I]FL is a relatively simple two-step process, as illustrated in Figure 2. A 5% yield of the product relative to the amount of

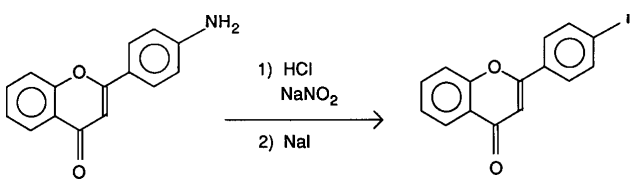

FIGURE 2. Synthesis of [^{125}I] 4′-iodoflavone.

iodine utilized in the reaction was obtained. The purity and identity of the radioactive 4′-iodoflavone was confirmed by thin-layer chromatography in several different solvent systems (data not shown). Thus we have synthesized an AhR ligand, [125I]FL, that has the same radiospecific activity as [125I]Br_2DpD, which would allow a direct comparison to be made between AhR complexes bound to each radioligand.

Use of [125I]FL to Examine the Liganded Mouse AhR

Several different methods were tested to obtain specific binding of [125I]FL to AhR using Hepa 1 cytosolic protein. Although the analysis involving the use of hydroxyapatite to remove excess ligand [27] gave much higher values for specific binding than the method using protamine sulfate precipitation [28], neither method was adequate for determining ligand saturation of the AhR because of high nonspecific binding. It was determined that receptor binding, followed by removal of excess ligand with charcoal, yielded acceptable results when combined with sucrose gradient sedimentation analysis. However, the charcoal concentrations must be optimized to prevent stripping of bound ligand while still efficiently removing excess ligand; the optimal concentration of charcoal was determined to be 0.24 mg charcoal/mg protein. The utility of [125I]FL for use in saturation binding experiments was not determined.

To fully demonstrate specific binding of [125I]FL to the AhR using Hepa 1 cytosol, we utilized cytosol prepared from the mutant cell line c4/1 [29]. As this mutant expresses AhR that cannot bind ligand, incubation of c4/1 cytosol with [125I]FL would result in detection of primarily nonspecific binding. A ~9 S peak was not observed following incubation of [125I]FL with c4/1 cytosolic protein (data not shown). As shown in Figure 3A, incubation of [125I]FL with cytosolic extracts followed by sucrose density analysis resulted in the detection of a ~9 S peak, the addition of a 20-fold molar excess of TCDF resulted in ablation of the 9 S peak. Incubation of Hepa 1 cytosolic protein with [125I]Br_2DpD with or without a 200-fold molar excess of TCDF resulted in the absence and presence of a 9 S peak, respectively (Figure 3B).

Detection of the 6 S Nuclear AhR Complex in the Nucleus Using [125I]FL

The ability of [125I]FL and [125I]Br_2DpD to mediate nuclear accumulation of the 6 S AhR complex in Hepa 1 cells was tested by adding an equal amount of each radioligand to cell culture. After 1 h of treatment

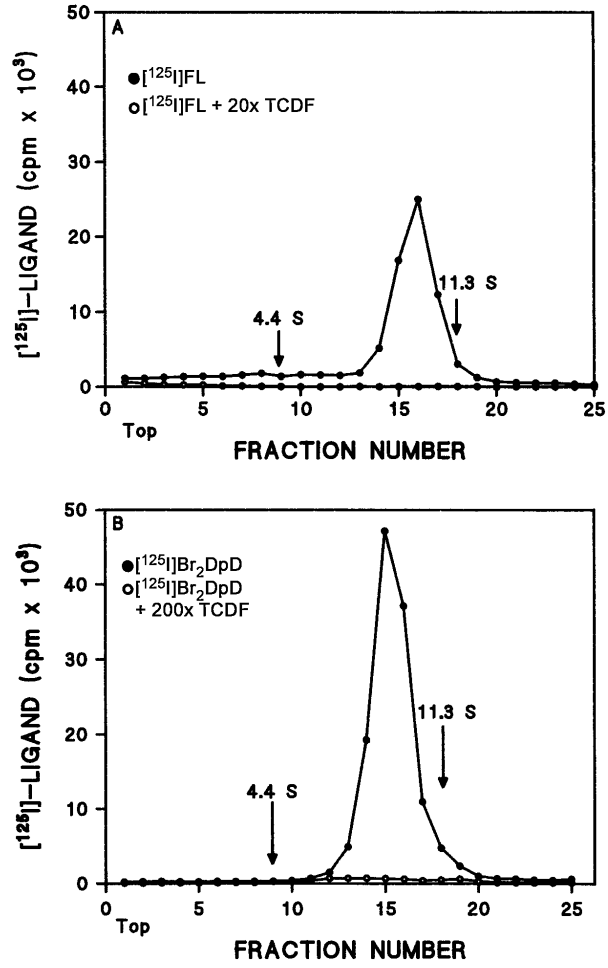

FIGURE 3. Sucrose density gradient analysis of specific binding of [125I]FL to the AhR using Hepa 1 cytosol as the receptor source. Hepa 1 (4.5 mg/mL) was incubated with 0.10 nM [125I]FL (A) or 0.10 pM [125I]Br_2DpD (B) in the absence or presence of either 20- or 200-fold molar excess of TCDF, followed by velocity sedimentation on 10–30% sucrose gradients, as described in Materials and Methods.

with each radioligand, nuclear extracts were isolated and subjected to sucrose gradient analysis. A predominantly 6 S radioactive peak was observed with both radioligands. The c4/2 cell line which does not express ARNT was utilized to demonstrate that the 6 S peak was absent when extracts from these cells were incubated with [125I]FL [30]. Thus, the 6 S radioactive peak observed in Hepa 1 cells does indeed represent the receptor complex. However, nuclear accumulation of [125I]Br_2DpD–AhR 6 S complexes in Hepa 1 cells was approximately threefold greater than that of [125I]FL-AhR after a 1-h incubation when incubated with equal amounts of each AhR ligand (Figure 4). This result also suggests that the greater amount of AhR–DNA complexes seen in Table 3 with 4′-iodoflavone treatment is due to the high concentration of 4′-iodoflavone used in the assay, but not due to an enhanced ability of

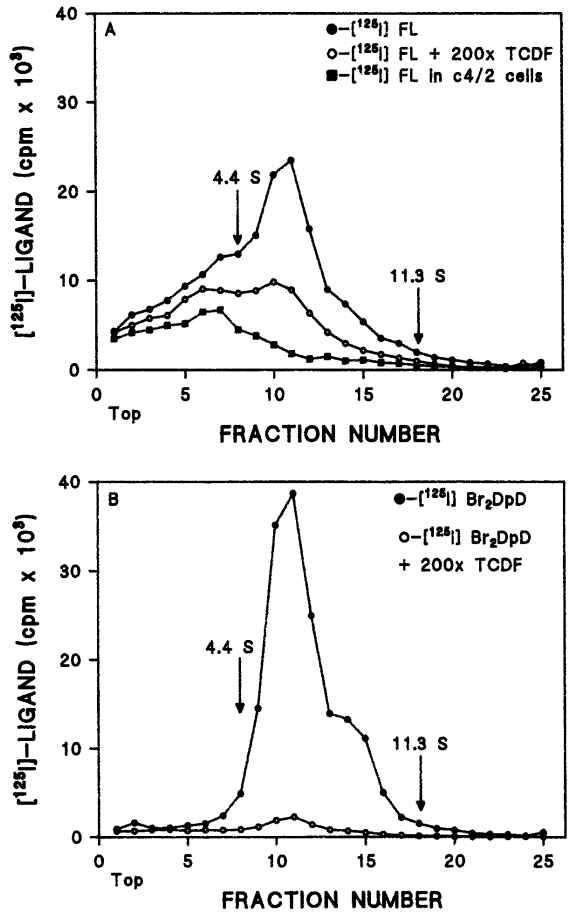

FIGURE 4. Nuclear uptake of [^{125}I]FL (A) or [^{125}I]Br_2DpD (B) in Hepa 1 cells. Either [^{125}I]FL or [^{125}I]Br_2DpD (0.52 nM) was added to nearly confluent Hepa 1 cells, with and without a 200-fold excess of TCDF, and incubated for 1 h. The cell line c4/2 was also incubated with [^{125}I]FL as shown in panel A. High salt nuclear extracts were prepared and analyzed by sucrose gradient centrifugation.

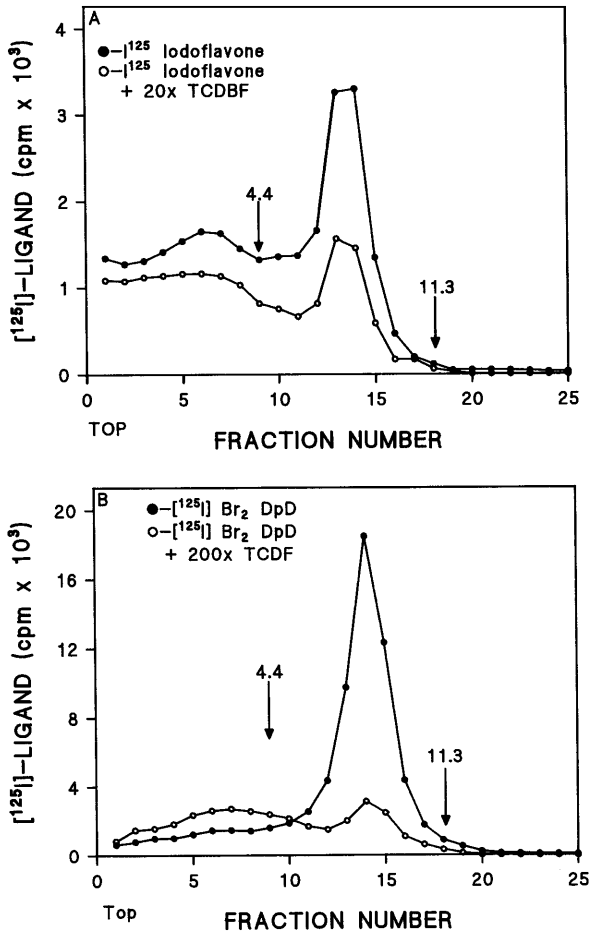

FIGURE 5. Sucrose density gradient analysis of specific binding of [^{125}I]FL (A) or [^{125}I]Br_2DpD (B) using HeLa cytosol. HeLa cytosol (3 mg/mL) was incubated with 0.10 nM of either [^{125}I]FL or [^{125}I]Br_2DpD in the absence or presence of excess TCDF. Following incubation, aliquots were removed and applied to 10–30% sucrose gradients as described in Materials and Methods.

4′-iodoflavone to cause the formation of AhR/ARNT complexes compared with TCDD on an equal molar basis. These results would indicate that both radioligands can be used to study the 6 S form of the AhR in cultured cells.

Use of [^{125}I]FL to Examine the Liganded Human AhR

Incubation of HeLa cytosolic protein with either radioligand resulted in a ~9.7 S peak following analysis by sucrose density centrifugation (Figure 5). Binding conditions were essentially the same as those described above using Hepa 1 cytosolic protein, except that the protein and charcoal concentrations were optimized to 3 mg/mL protein and 0.14 mg charcoal/mg protein. This data clearly indicates that [^{125}I]FL has sufficient affinity for the human AhR to be utilized to study this receptor. Analysis of the nuclear uptake of both radioligands in HeLa cells demonstrated that both compounds are efficiently transported to the nucleus. After 1-h exposure to radioligand, the radioligand–AhR complex was detected by the appearance of a ~6.7 S peak following sucrose density centrifugation analysis (Figure 6). No radioactive peak was detected following sucrose density centrifugation analysis of nuclear extracts from HeLa cells incubated with [^{125}I]FL in the presence of 100-fold M excess of either 4′-iodoflavone or 3-methylcholanthrene (results not shown). Interestingly, following the addition of equimolar concentrations (0.52 nM) of either radioligand to HeLa cells in culture, a 2.5-fold greater amount of [^{125}I]FL–AhR complex was recovered in the nuclear fraction (Figure 6). This is the opposite of what was observed in Hepa 1 cells and would suggest that [^{125}I]FL would be quite an effective tool to study the human AhR.

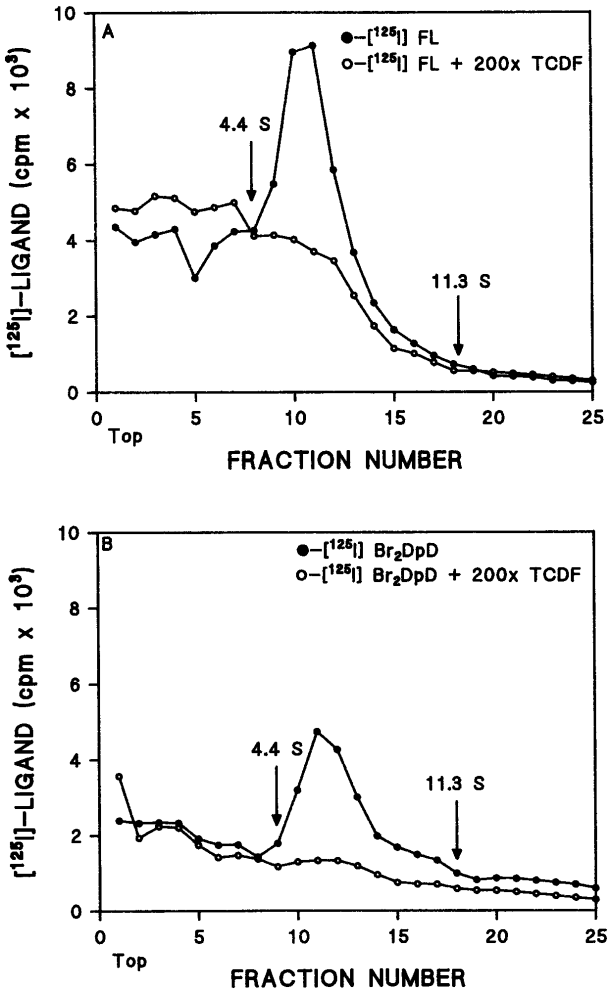

FIGURE 6. Nuclear uptake of [125I]FL (A) and [125I]Br_2DpD (B) in HeLa cells. Either [125I]FL or [125I]Br_2DpD (0.52 nM) was added to nearly confluent HeLa cells, with and without a 200-fold excess of TCDF, and incubated for 1 h. High salt nuclear extracts were prepared and analyzed by sucrose density gradients centrifugation. Typical results of three separate experiments are shown.

Iodoflavone–AhR Complex Exhibits a Rapid Rate of Nuclear Translocation

Considering that [125I]FL exhibited greater levels of 6 S AhR complex in HeLa cells and lower amounts of nuclear 9 S AhR complex in Hepa 1 cells as compared to levels observed with the radioactive dioxin analogue, we wanted to explore whether there were temporal differences in the rate of accumulation of each radioactive ligand-bound AhR complex. The amount of 6 S and 9 S AhR complexes bound to ligand in the nuclei of Hepa 1 cells was assessed at 15, 30, 60, and 120 min. Data obtained from sucrose gradient analysis is shown in Figure 7 for nuclear extracts from Hepa 1 cells at 15 and 60 min and revealed that twice as much [125I]FL-bound AhR complexes are present in the nucleus compared with [125I]Br_2DpD–AhR complexes after 15 min (Figure 7), while at 1 h approximately threefold more [125I]Br_2DpD–AhR complexes are observed. This result would suggest that 4′-iodoflavone quickly enters the cell and binds to the AhR and induces nuclear accumulation faster than the higher affinity dioxin analogue, which has lower aqueous solubility. Interestingly, plotting the total amount of nuclear receptor versus time reveals that the amount of [125I]FL–AhR complexes observed is maximal at 15 min (Figure 8). In contrast, the maximum amount of [125I]Br_2DpD–AhR complexes in the nucleus occurred at 60 min. To further explore the apparent rapid rate of 4′-iodoflavone-mediated nuclear AhR uptake, COS 1 cells were cotransfected with mAhR-YFP and XAP2 constructs. XAP2 was coexpressed to ensure total cytoplasmic localization of the AhR, as observed previously [31]. Transfected cells were treated with TCDD or 4′-iodoflavone and were examined at a series of time points for the level of mAhR-YFP found in the nucleus. Cells were scored for either predominantly nuclear or cytoplasmic localization of mAhR-YFP. The results clearly indicated that 22% of the cells treated with 4′-iodoflavone for 15 min exhibited predominantly nuclear localization of mAhR-YFP (Figure 9). In contrast, examination of cells treated with TCDD revealed that only 2% of the cells exhibited predominantly nuclear localization of mAhR-YFP. This data confirms and extends the assertion that 4′-iodoflavone induces rapid translocation into the nucleus and subsequent heterodimerization with ARNT.

DISCUSSION

A number of recent studies have established that there are ligand-dependent differences in the three-dimensional structure of liganded nuclear receptors. These studies also suggest that the conformational change induced by ligand binding should not be thought of as a switch but instead as a rheostat that can assume a number of conformations. For example, both tamoxifen and raloxifene are estrogen receptor antagonists in breast tissue. However, tamoxifen increases the incidence of endometrial cancer, while raloxifene does not [32]. These results could be explained by differential recruitment of coregulators by the ER bound by each antagonist and/or differences in expression of coregulators in different cell types [33]. Studies utilizing various LXXLL motif peptides derived from coactivators were tested for their ability to interact with the estrogen receptor in the presence of four distinct ligands [34,35]. The data suggests that each ligand differs in its ability to enhance binding of a series of LXXLL peptides. These results support the assertion that nuclear

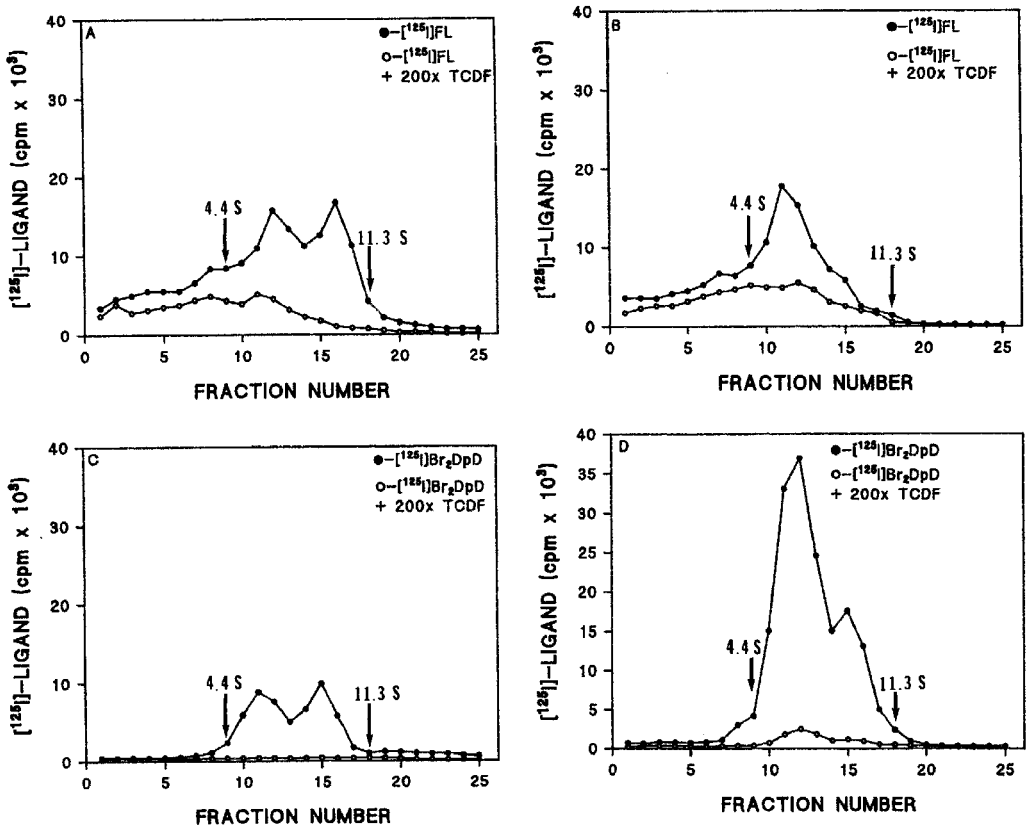

FIGURE 7. Nuclear accumulation of either [^{125}I]FL or [^{125}I]Br_2DpD in Hepa 1 cells. Either radioligand (0.52 nM) was added to nearly confluent Hepa 1 cells, in the absence or presence of a 200-fold excess of TCDF. At 15, 30, 45, 60, and 120 min following radioligand addition, the cells were harvested and high salt nuclear extracts were prepared. The samples were analyzed by sucrose gradient centrifugation as described in Materials and Methods. Shown in the figure are [^{125}I]FL–AhR nuclear extract complexes from 15 min (A) and 60 min (B) after radioligand addition and [^{125}I]Br_2DpD–AhR nuclear complexes from 15 min (C) and 60 min (D) following radioligand addition. Results representative of three separate experiments are shown.

receptors such as the estrogen receptor can assume multiple conformations dependent on the ligand occupying the ligand-binding pocket. From these observations it is logical to hypothesize that the conformation of the AhR/ARNT heterodimer is dependent on the structure of the ligand bound to the AhR. Firm support for this hypothesis has been recently obtained examining the promoter region of the Bax gene [36]. Benzo(a)pyrene induces Bax mRNA, while TCDD treatment fails to enhance Bax mRNA levels in vivo. The promoter region of the Bax gene contains a DRE that is functional in the presence of a Benzo(a)pyrene/AhR/ARNT complex, while the TCDD/AhR/ARNT complex is not capable of binding to this DRE [36]. This is the first demonstration of a ligand-dependent difference in the ability of the AhR/ARNT complex to bind to a given DRE. This result suggests that an AhR ligand can actually influence the conformation of the AhR/ARNT heterocomplex and influence response element recognition.

Some biological responses mediated by the AhR have been shown to have ligand-specific differences.

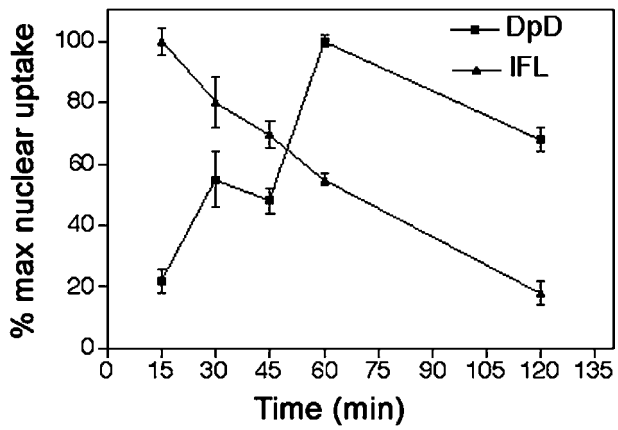

FIGURE 8. Summary of time course analysis of the total amount of AhR in either the 6 S or 9 S forms present in nuclear extracts upon treatment of Hepa 1 cells with [^{125}I]FL or [^{125}I]Br_2DpD in cell culture. This figure is a summary of the data presented in Figure 7 as well as additional time points. Nuclear uptake of [^{125}I]FL or [^{125}I]Br_2DpD bound to the 6 S and 9 S forms of the AhR in Hepa 1 cells was assessed as outlined in Figure 7 legend. Data was collected at 15, 30, 45, 60, and 120 min. Each time point was performed in triplicate.

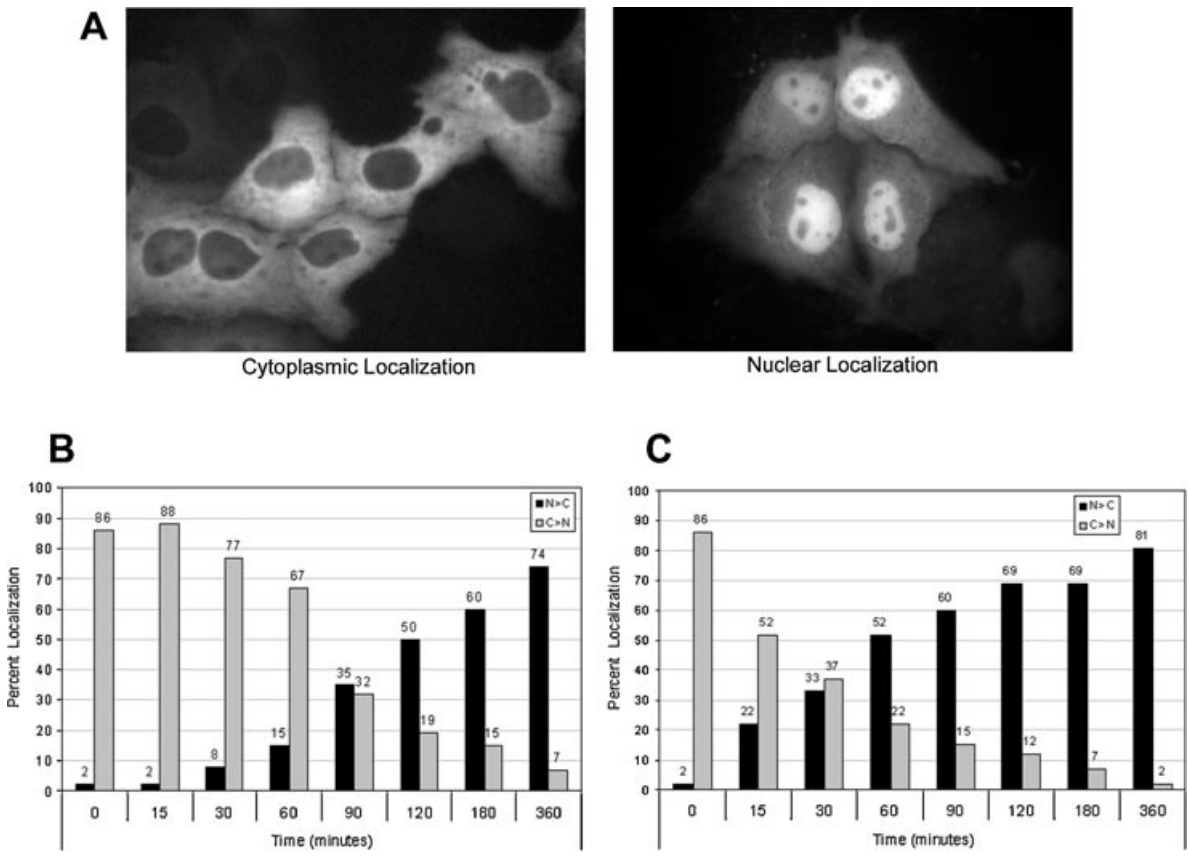

FIGURE 9. Time-dependent nuclear accumulation of mAhR-YFP mediated by iodoflavone or TCDD. COS 1 cells were grown in 6-well dishes and transfected with 500 ng of pEYFP-mAhR and 1.5 μg of pCI. Cells were treated with either 10 nM TCDD or 10 μM iodoflavone and then were visually inspected by fluorescence microscopy. At a series of time points post-ligand treatment, at least 100 cells were inspected and scored for whether they exhibited predominantly cytoplasmic or nuclear localization of mAhR-YFP. (A) An example of predominantly cytoplasmic and nuclear localization of mAhR-YFP in COS 1 cells; (B) Summary of scoring results from inspection of mAhR-YFP localization over time after TCDD treatment. (C) Summary of scoring results from inspection of mAhR-YFP localization over time after iodoflavone treatment.

For example, 4′-bromoflavone and 5,6-benzoflavone have similar binding affinities for the AhR. However, 4′-bromoflavone was substantially less effective in producing AhR-mediated keratinization of keratinocyte cells [37]. This is an interesting observation upon considering the similiarities of the two structures. Rats treated with TCDD, but not βNF, showed an elevation of cytosolic AhR levels in the liver [38,39]. Certain biochemical properties of liganded AhR also appear to be dependent on the type of agonist bound. For example, a comparison was made of the molecular properties of 2,3,7,8-[^{3}H]TCDF rat receptor complex with that of 2,3,7,8-[^{3}H]TCDD. Receptor equilibrium binding with 2,3,7,8-[^{3}H]TCDF resulted in the presence of both a ~9 S and a ~5 S peak after incubation in low salt followed by sucrose density analysis. However, only the ~9 S peak was recovered while using [^{3}H]TCDD as the radioligand [40] which may indicate that occupation with [^{3}H]TCDF better facilitates Hsp90 dissociation. Previous studies using five polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners as AhR ligands have shown that the accumulation of the nuclear form of ligand–receptor complex could be linearly correlated with the corresponding induction of cytochrome P4501A1 [41]. This led the authors to suggest that the level of nuclear receptor complexes is critical in determining the ligand potency as inducing agents. Indeed, our results here would suggest that 4′-iodoflavone is efficient at inducing nuclear accumulation of the AhR as well as inducing EROD activity.

AhR agonists typically studied represent two classes of compounds: polycyclic aromatic hydrocarbons (e.g. benzo[a]pyrene) and polycyclic aromatic dibenzo-*p*-dioxins (e.g. TCDD), with the latter being the most studied. We have synthesized [^{125}I]FL to study the AhR's interaction with a ligand representing the class of compounds known as the flavones. 4′-Iodoflavone displayed a similar AhR affinity as βNF (Figure 2). This was demonstrated by the IC_{50} values of 12.2 and 8.45 nM determined for iodoflavone and

βNF, respectively. Several structurally distinct groups of compounds have been shown to bind with relatively high affinity to the AhR and mediate a corresponding induction of cytochrome P450IA1 [12]. Flavones, such as βNF and 4′-bromoflavone, bind with relatively lower affinity to the AhR in rodents than TCDD ([25], Figure 2), yet they are potent inducers of AHH activity [13,14]. Although less TCDD is required to obtain a maximal AHH induction response in Hepa 1 cells, βNF gave a higher maximal response than that observed for TCDD [42]. In rat liver cells, 4′-bromoflavone induced AHH activity to a greater level than did 3-methylcholanthrene [13]. Collectively, these studies suggest that AhR agonist vary in their efficacy to induce a biochemical response.

Certain cytosolic steroid hormone receptors (e.g. the glucocorticoid receptor) are complexed with Hsp90 in a non-DNA binding form [43]. Activation to a DNA binding form results following agonist binding and dissociation of Hsp90 from the receptor [44]. However, occupation with the antagonist RU 486 appears to stabilize the glucocorticoid receptor–Hsp90 complex in the cytosol [45], preventing nuclear translocation and subsequent DNA binding [46,47]. It has been postulated that a primary mechanism of action of a steroid receptor antagonist is stabilization of the oligomeric non-DNA binding form (see Ref. [48] for review). It does not seem unreasonable to suggest that agonists with unequal receptor affinities may differ in their ability to cause receptor Hsp90 dissociation. The result of this could be either differing amounts of receptor complex accumulating in the nucleus, or receptor complexes which differ in affinity for DNA, or both. Interestingly, the initial nuclear accumulation of the [^{125}I]FL–receptor complex was three times more than that of the [^{125}I]Br_2DpD complex. Despite the fact that [^{125}I]Br_2DpD and [^{125}I]FL are both agonist, affinity alone would appear not to be the only factor in determining the rate of transformation to the DNA binding (6 S form) of the AhR in cell culture. Several mechanisms may explain the ligand-specific differences in AhR activation observed in this study. For example, an AhR ligand's hydrophobicity and solubility in aqueous buffers would influence its ability to partition across membrane and bind to the AhR in the cytoplasm. Another possibility is that, upon AhR–ligand binding, the structure of the ligand may cause a conformational change in the receptor, thereby facilitating the dissociation of the Hsp90 dimer from the AhR, which is an essential step prior to AhR/ARNT dimerization. The ability to induce hsp90 dissociation from the AhR by a given ligand would be dependent on the properties of that ligand not solely on its affinity for the AhR. Also, it is quite possible that the ligand may be important in enabling the AhR/ARNT heterodimer to bind to actual DNA enhancer elements, as opposed to binding to other nuclear components. Finally, the biological potency of a ligand may be dependent on the length of time the AhR–ligand complex resides in the nucleus and interacts with DNA. Thus, ligand may be an important determinant of the half-life of AhR in the nucleus. The synthesis of [^{125}I]FL presented here will enable further studies to examine the mechanism of enhanced temporal uptake of AhR mediated by iodoflavone compared with TCDD.

Many features of the AhR in rodents and humans are similar, but distinct differences do exist. These include the higher molecular weight form [49] and the ability of molybdate to stabilize the human receptor [9]. Human breast cell lines have been shown to be inducible by TCDD, which results in an increase in mRNA of cytochrome P450IA1 [50]. Upon exposure to various AhR agonist these cell lines have shown structure–function relationships similar to that found in rodent cell lines [51]. The results in the present study confirm previous reports that the cytosolic form of the human AhR ligand-complex sediments at ∼9 S and the nuclear form sediments at ∼6 S as is also described for the AhR obtained from rodent sources ([13,51]; Figures 3–6). This evidence suggests that the human AhR undergoes a transformation and nuclear translocation process similar to that observed in rodent systems, easily detected due to the high specific activity of the radioligands used in this study. [^{125}I]FL introduced in this work should prove useful as a tool to further study the human AhR as it has high radiospecific activity and is easily synthesized.

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