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Transient induction of cytochromes P450 1A1 and 1B1 in MCF-7 human breast cancer cells by indirubin

Barbara C. Spink, Mirza M. Hussain, Barbara H. Katz, Leslie Eisele, David C. Spink*

New York State Department of Health, Wadsworth Center, Albany, NY 12201-0509, USA

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Abstract

The aryl hydrocarbon receptor (AhR), when activated by exogenous ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), regulates expression of several phase I and phase II enzymes and is also involved in the regulation of cell proliferation. Several studies suggest that endogenous AhR ligand(s) may exist. One putative endogenous ligand is indirubin, which was recently identified in human urine and bovine serum. We determined the effect of indirubin in MCF-7 breast cancer cells on induction of the activities of cytochromes P450 (CYP) 1A1 and 1B1, as measured by estradiol and ethoxyresorufin metabolism, and on induction of the CYP1A1 and CYP1B1 mRNAs. With 4-hr exposure, the effects of indirubin and TCDD at 10 nM on CYP activity were comparable, but the effects of indirubin, unlike those of TCDD, were transitory. Indirubin-induced ethoxyresorufin-*O*-deethylase activity was maximal by 6–9 hr post-exposure and had disappeared by 24 hr, whereas TCDD-induced activities remained elevated for at least 72 hr. The effects of indirubin on CYP mRNA induction were maximal at 3 hr. Indirubin was metabolized by microsomes containing cDNA-expressed human CYP1A1 or CYP1B1. The potency of indirubin was comparable to that of TCDD in a CYP1B1-promoter-driven luciferase assay, when MCF-7 cells were co-exposed to the AhR ligands together with the CYP inhibitor, ellipticine. Thus, if indirubin is an endogenous AhR ligand, then AhR-mediated signaling by indirubin is likely to be transient and tightly controlled by the ability of indirubin to induce CYP1A1 and CYP1B1, and hence its own metabolism.

Keywords: Aryl hydrocarbon receptor; Cytochrome P450; CYP1A1; CYP1B1; Indirubin; Real-time PCR

1. Introduction

The AhR was originally identified and characterized as a xenobiotic receptor, and it has been extensively studied in this context. Many of the toxic effects of environmental contaminants, including polycyclic aromatic hydrocarbons and polyhalogenated dioxins, furans, and biphenyls, are mediated by binding to and activation of the AhR. In its unliganded state, the AhR is found in the cytoplasm in a complex with two molecules of heat shock protein 90 and additional cellular chaperones, ARA9 (also known as AIP1 or XAP2) and p23 (reviewed in [1]). Binding of ligand results in dissociation of this complex and translocation of the AhR to the nucleus, where, together with its dimerization partner, the AhR nuclear translocator, it binds to DREs in the 5′-regulatory regions of a number of genes, thereby enhancing the rates of transcription. These genes, which are often referred to as the Ah gene battery, encode the phase I enzymes, CYP1A1, CYP1A2, and CYP1B1, as well as several phase II enzymes that are involved in the metabolism of xenobiotics and endogenous compounds [2]. The regulation of the Ah gene battery, however, appears to represent only a subset of the physiologic roles of the AhR.

When activated by an exogenous ligand such as TCDD, the AhR is known to affect cell proliferation and cell-cycle control in a cell-specific manner [3]. Ligand-activated AhR was recently shown to interact with the retinoblastoma protein, and to delay cell-cycle progression in 5L hepatoma cells [4] and MCF-7 breast cancer cells [5]. Activation of the AhR by TCDD resulted in suppression of cellular proliferation of 5L hepatoma cells by inducing the

* Corresponding author. Tel.: +1-518-486-2532; fax: +1-518-486-1505.
E-mail address: spink@wadsworth.org (D.C. Spink).

Abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DRE, dioxin response element; E_2, 17β-estradiol; EROD, ethoxyresorufin-*O*-deethylase; OR, NADPH cytochrome P450 oxidoreductase; HPLC, high-performance liquid chromatography; $MeOE_2$, methoxyestradiol; SEM, standard error of the mean; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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cell-cycle inhibitor, $p27^{Kip1}$ [6]. In MCF-7 cells exposed to benzo[*a*]pyrene, BRCA-1 protein expression was reduced, concomitant with accumulation of p53 and with cell-cycle arrest [7]. Liganded AhR increased the mRNA levels for the immediate-early response genes, *fos* and *jun*, in mouse hepatoma cells [8].

A number of studies have reported that AhR-mediated processes occur in the absence of exogenous AhR ligands. Elevated AhR expression led to increased rates of proliferation of mouse Hepa 1c1c7 hepatoma [9] and human A549 lung [10] cells. Embryonic fibroblasts derived from AhR-null mice showed reduced rates of cell proliferation compared with fibroblasts from controls [11], and primary hepatocyte cultures from AhR-null mice had elevated numbers of cells undergoing apoptosis [12]. CYP1A1 was shown to be induced by altered cell culture conditions such as suspension [13,14] and hydrodynamic shear [15], and to be induced more than 100-fold during early embryogenesis of the mouse [16]. In HeLa cells cultured without exposure to exogenous ligands, approximately 16% of the AhR pool was found to be tightly associated with the nuclear fraction, suggesting that it is in an activated state and is involved in transcriptional regulation [17]. These studies suggest that, in addition to the known xenobiotic ligands, there may exist an endogenous ligand(s) for the AhR that mediates autocrine or paracrine functions.

There have been numerous investigations with the goal of identifying endogenous AhR ligands [18]. Recently, indirubin and indigo, two high-affinity AhR ligands [19], were isolated from human urine and fetal bovine serum and were shown to elicit AhR-mediated responses [20]. The potency of indirubin exceeded that of TCDD by about 50-fold in a yeast AhR-dependent reporter assay. Although indirubin is not known to be a product of metabolism in any animal species, it is formed spontaneously by the dimerization of oxidation products of indoxyl, a product of hepatic CYP-catalyzed metabolism of indole [21,22]. In this study, we evaluated the efficacy of indirubin in MCF-7 breast cancer cells in the AhR-regulated transcription of two extra-hepatic, phase I metabolic enzymes, CYP1A1 and CYP1B1. We found that indirubin is a potent inducer of CYP1A1 and CYP1B1, but is rapidly metabolized by the enzymes it induces.

2. Materials and methods

2.1. Chemicals

TCDD was purchased from Cambridge Isotope Laboratories. Estrogen metabolite standards were from Steraloids. Type H-2 β-glucuronidase/aryl sulfatase, NADPH, indigo, 3-indoxyl acetate, isatin, α-naphthoflavone, and DMSO were from Sigma–Aldrich. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide was from Pierce. Indirubin was prepared by condensation of 3-indoxyl acetate and isatin under N_2 in the presence of sodium carbonate as described [23]. The structures of indirubin and TCDD are shown in Fig. 1.

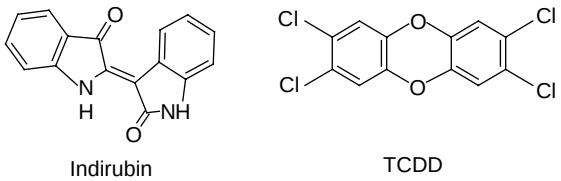

Fig. 1. The structures of indirubin and TCDD.

2.2. Culture of MCF-7 cells

Stock cultures of the MCF-7 cells were maintained at 37° in a humidified atmosphere with 5% CO_2 and DF_5 medium, consisting of DMEM supplemented with 5% (v/v) fetal bovine serum (Hyclone Laboratories), 10 mM non-essential amino acids, 2 mM L-glutamine, 10 μg/L insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin. For experimental protocols the medium used was DC_5, which differed from DF_5 medium in that it contained 5% bovine calf serum (Cosmic calf serum; Hyclone Laboratories) rather than fetal bovine serum, and in that phenol red was omitted.

2.3. Effects of indirubin on E_2 metabolism catalyzed by CYP1A1 and CYP1B1

The effects of indirubin on the metabolism of the most potent, naturally occurring estrogen, E_2, were determined by methods previously described [24,25]. Briefly, confluent cultures of cells in 6-well plates were exposed to the solvent vehicle, DMSO, at 0.1% (v/v), or to 10 nM TCDD, or to 0.1–10 nM indirubin in fresh DC_5 medium, either concurrently with 1 μM E_2 for 6-hr exposure, or without E_2 for 24 hr, followed by replacement of the medium with medium containing 1 μM E_2 for 6 hr. Medium was subsequently treated with β-glucuronidase/aryl sulfatase to hydrolyze conjugates of the estrogen metabolites, and was subjected to solid-phase extraction with methylene chloride elution; extracts were evaporated to dryness under N_2. Trimethylsilyl derivatives of the E_2 metabolites were prepared by reaction with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide in pyridine for 30 min at 60°, and were analyzed by gas chromatography–mass spectrometry with stable-isotope dilution. Total protein content was determined by use of the bicinchoninic acid assay kit from Pierce.

2.4. Quantitation of CYP1A1, CYP1B1, and 36B4 mRNAs by real-time PCR

Confluent MCF-7 cultures were exposed to 0.1% DMSO (v/v) or 10 nM indirubin in DC_5 for 1–12 hr. Due to the transient AhR activation that results from provision of

fresh medium to cell cultures [26], exposure of MCF-7 cells to TCDD and indirubin was performed with conditioned medium by removal of half of the medium from the wells, combination of it with twice the desired final levels of DMSO or indirubin, and addition of the medium back to the wells. After exposure for varying times, total RNA was isolated, and it was primed with oligo-dT and reverse-transcribed with Superscript II reverse-transcriptase followed by treatment with RNaseH (Invitrogen) as described previously [27]. Portions of the CYP1A1 and CYP1B1 cDNAs were amplified by PCR by use of the primers previously published [27], and CYP1A1 and CYP1B1 mRNA levels were normalized to the mRNA levels of the housekeeping gene, *36B4*, which encodes acidic ribosomal phosphoprotein PO [28]. For amplification of a 277-bp fragment of the 36B4 cDNA (GenBank accession number M17885; nucleotides 305–581), the primers were: 5′-GAAACTGCTGCCTCATATCC-3′ (forward) and 5′-TCCCACTTTGTCTCCAGTC-3′ (reverse). All amplified cDNAs spanned at least two exons, and their sequences were confirmed by nucleotide sequencing with a Perkin-Elmer Biosystems ABI PRISM 377XL automated DNA sequencer. Real-time PCR was carried out with FastStart DNA Master SYBR Green I system from Roche Molecular Biochemicals using 3 mM $MgCl_2$, 0.5 μM of each primer, and cDNA from 50 ng reverse-transcribed RNA. The cycling conditions were: initial denaturation at 95° for 10 min, followed by 45 cycles of denaturation at 95° for 15 s, annealing at 65° for 5 s, extension at 72° for a period (in s), equal to the number of base pairs of amplified product divided by 25, and a hold for 5 s at 83° for CYP1A1 and 36B4 target cDNAs, or at 81° for CYP1B1 target cDNA, before fluorescence data acquisition. Complementary DNA was quantified by comparison of the number of cycles required for amplification of unknowns with those required for amplification of a series of cDNA standards of known concentration. These standards were prepared by amplification of the target cDNAs, purification by agarose gel electrophoresis, and recovery of them by use of the QIAquick gel extraction kit (Qiagen). The concentrations of the purified cDNA standards were determined by agarose gel electrophoresis and densitometry relative to low mass ladder standards (Invitrogen). The standards were stored at −80° in 5 mM Tris buffer, pH 8, containing 50 μg/mL glycogen.

2.5. Assay of EROD activity

EROD assays were performed by a modification of the 96-well plate procedure of Donato *et al.* [29]. Confluent cultures were treated for varying time periods with 0.1% (v/v) DMSO, indirubin, or TCDD in 200 μL DC_5 medium prior to the EROD assay. For these treatments, half of the medium was removed from the wells, compounds were mixed with the conditioned medium at twice the desired final concentration, and the medium was then returned to the wells. EROD activity was assayed by replacement of the medium with the substrate, 2.2 μM ethoxyresorufin, and 10 μM dicumarol in fresh DC_5 medium (100 μL/well). After 30 min at 37°, 75 μL of the medium was transferred to a white, opaque 96-well plate (Bioworld Lab Essentials). Ethanol (200 μL/well) was added to stop the reaction, and the plate was centrifuged at 1500 *g* for 10 min. Blanks were prepared by exposure of untreated cells to 10 μM α-naphthoflavone, a CYP1A1 inhibitor, for 30 min prior to and during the EROD assay. Fluorescence was measured in a Fusion Universal Microplate Analyzer (Packard) using 535-nm excitation and 590-nm emission filters. Resorufin production was linear for 60 min, and the fluorescence of the reaction was compared with a standard curve for resorufin prepared in DC_5 medium and ethanol, a curve which was linear to 40 pmol of resorufin.

2.6. Microsomal incubations and measurement of indirubin by HPLC

The incubation of indirubin with *Sf9* insect cell microsomes containing human CYP1A1 or CYP1B1 co-expressed with human OR (Gentest) was performed in 250 μL of 0.1 M sodium phosphate buffer, pH 7.4, containing 10 μM indirubin, 5 mM $MgCl_2$, and 3 pmol CYP1A1 or CYP1B1, or control microsomes. Reaction mixtures were pre-incubated at 37° for 5 min and were initiated by the addition of 1.4 mM NADPH. After 30 min, reactions were extracted twice with 3-mL portions of ethyl acetate. Indigo (25 nmol) was added as the internal standard, and the combined organic phases were evaporated under N_2 until the samples were reduced in volume to about 500 μL. Indirubin and indigo were analyzed by reverse-phase HPLC using a modification of the procedure of Maugard *et al.* [30]. Resolution of indirubin and indigo was achieved on a Vydac C_{18} column (4.6 mm i.d. × 250 mm, 5 μm particle size) using a Waters 625LC system with 717+ autosampler and 996 photodiode array detector. The flow rate was 1 mL/min using a gradient made of solvent A (H_2O/0.2% trifluoroacetic acid) and solvent B (CH_3OH/0.2% trifluoroacetic acid) as follows: from 0 to 10 min, change from 85% A/15% B to 15% A/85% B; from 10 to 20 min, hold at 15% A/85% B; from 20 to 25 min, change to 85% A/15% B; and from 25 to 30 min, hold at 85% A/15% B. Indirubin and indigo were detected by their absorbance at 552 nm and were further identified by their spectra [30].

2.7. Assay of CYP1B1 promoter activity in MCF-7 cells transiently transfected with a luciferase construct

A CYP1B1 promoter firefly luciferase construct, p1B1Fluc [25], containing the region of the CYP1B1 promoter corresponding to nucleotides −1141 to +5 [31] cloned into the pGL3 Basic vector (Promega), was used in transient transfection experiments with MCF-7

cells grown in 24-well plates. Briefly, 180 ng of p1B1Fluc, 20 ng of the pRL-CMV *Renilla* luciferase control vector (Promega), and 300 ng of carrier pBluescript SK vector (Stratagene) were transiently transfected into MCF-7 cells at 90% confluence using 2 μL LipofectAMINE 2000 (Invitrogen) in 100 μL DMEM per well as described [25]. After 12 hr, the medium was replaced with 250 μL of conditioned DC_5 medium containing either DMSO vehicle only or increasing concentrations of indirubin or TCDD, with or without 100 nM ellipticine, a type II CYP inhibitor [32]. This conditioned DC_5 medium was prepared by exposure of untreated MCF-7 cells in T-75 flasks to DC_5 medium for 3 days. After treatment for 12 hr, the cultures were assayed for luciferase activity by use of the Dual-Luciferase Reporter 1000 Assay System (Promega).

2.8. Statistical analysis

All determinations that were made in triplicate or quadruplicate are reported as the mean ± SEM. Determinations made in duplicate are reported as the mean ± range. Treatment groups were subjected to one-way ANOVA and Bonferroni's test for multiple comparisons; significance was established at the level of $P < 0.05$. For determination of EC_{50} values, dose–response data were fitted to the four-parameter sigmoidal function by use of SigmaPlot (SSPS).

3. Results

3.1. Effects of indirubin on estrogen metabolism in MCF-7 Cells

The effects of exposure to indirubin or TCDD on formation of 2- and 4-$MeOE_2$, which reflect CYP1A1 and CYP1B1 activity, respectively [33,34], are shown in Fig. 2A. After exposure for 24 hr to 0.1% (v/v) DMSO, 10 nM TCDD, or varying concentrations of indirubin, the cells were assayed for $MeOE_2$ formation by replacement of the medium with medium containing 1 μM E_2 for 6 hr. Exposure to TCDD caused 22.8- and 9.0-fold increases in the rates of 2-$MeOE_2$ and 4-$MeOE_2$ formation, respectively, relative to rates for the DMSO control. No elevated rates of E_2 metabolism were elicited by indirubin under these conditions. Given the possibility that induction of CYP1A1 and CYP1B1 by indirubin may be transient, MCF-7 cells were exposed to 0.1% (v/v) DMSO, 10 nM TCDD, or 0.1–10 nM levels of indirubin, concurrently with 1 μM E_2, during the 6-hr assay for 2- and 4-$MeOE_2$ formation. As seen in Fig. 2B, under these conditions, 10 nM indirubin caused a 2.8-fold increase in the rate of 2-$MeOE_2$ formation relative to the rate in the DMSO control; this indirubin-induced rate was not significantly different from the TCDD-induced rate. The rates of 4-$MeOE_2$ formation induced by indirubin or TCDD were not significantly different from the rate of the DMSO control.

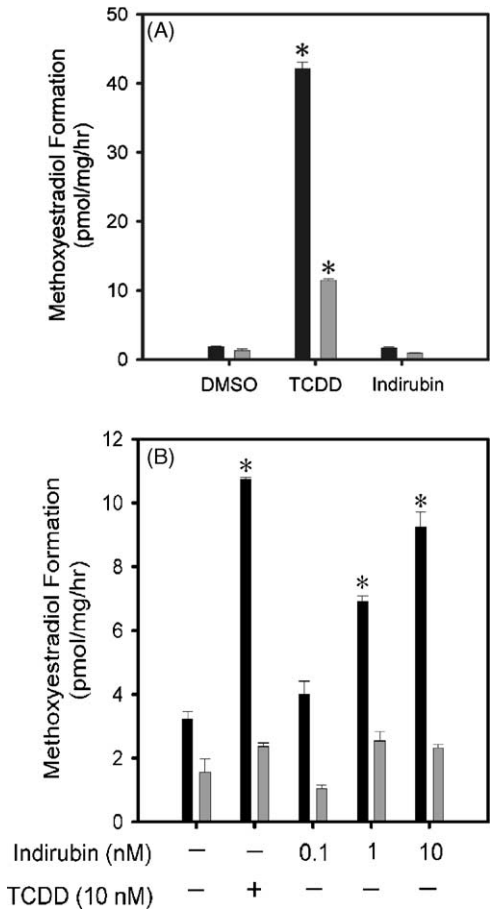

Fig. 2. Effects of exposure to TCDD or indirubin on 2- and 4-$MeOE_2$ formation in MCF-7 cells. In part (A), cultures were exposed to the solvent vehicle (0.1% DMSO), 10 nM TCDD, or 10 nM indirubin for 24 hr. Medium was then replaced with medium containing 1 μM E_2 for assay of 2-$MeOE_2$ and 4-$MeOE_2$ formation for 6 hr as described in Section 2. In part (B), cultures were exposed for 6 hr to the solvent vehicle (0.1% DMSO), 10 nM TCDD, or 0.1–10 nM indirubin, concurrently with 1 μM E_2, for assay of 2-$MeOE_2$ and 4-$MeOE_2$ formation. Rates of formation of 2-$MeOE_2$ (filled bars) and 4-$MeOE_2$ (gray bars) are shown as the mean ± SEM of determinations from three cultures of cells. An asterisk (*) indicates significantly different from DMSO control ($P < 0.001$).

3.2. Effects of indirubin on expression of the CYP1A1 and CYP1B1 mRNAs

The effects of exposure to 10 nM indirubin or vehicle for 1–12 hr on the levels of CYP1A1, CYP1B1 mRNAs normalized to those of 36B4 are shown in Fig. 3. The highest levels of the CYP mRNAs were observed at 3 hr post exposure, with 9.6- and 3.2-fold induction of the CYP1A1 and CYP1B1 mRNAs, respectively, over the levels in the DMSO control. CYP1A1 and CYP1B1 mRNAs returned to basal levels by 12 hr. Initially, levels of the 36B4, CYP1A1 and CYP1B1 mRNAs were 59.5± 1.87 amol/μg, 1.1± 0.03 amol/μg and 7.0 ± 0.23 amol/μg total RNA, respectively.

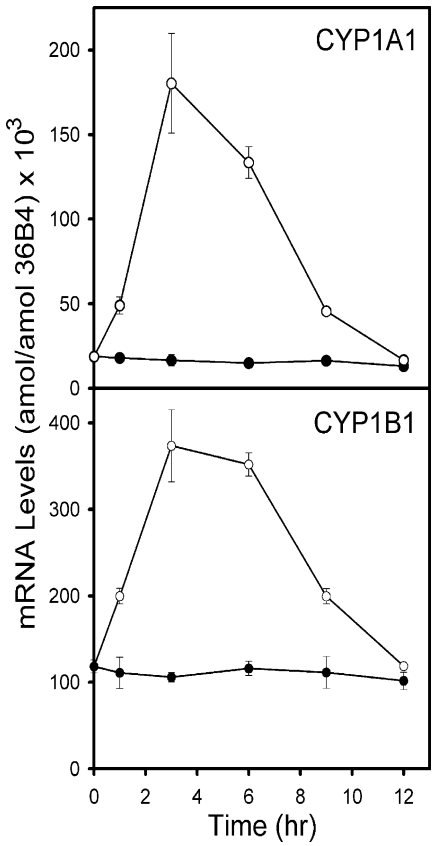

Fig. 3. Time course of the effects of indirubin on CYP1A1 and CYP1B1 mRNA levels. MCF-7 cultures were exposed to 10 nM indirubin (○) or 0.1% (v/v) DMSO (●) over a 12-hr period. At the indicated times, total RNA was isolated and reverse-transcribed, and the cDNA was amplified by real-time PCR for quantitative analysis as described in Section 2. The levels of CYP1A1 and CYP1B1 mRNA are expressed relative to 36B4 mRNA levels; they represent the mean ± SEM of determinations from three cultures of cells for indirubin values, or the mean ± range of determinations from two cultures of cells for DMSO values.

3.3. Effects of indirubin on EROD activity

Since the effect of indirubin on CYP induction, as assayed by E_2 metabolism, was greater for CYP1A1 than for CYP1B1, and since the 6-hr time period required for this assay was not ideal for measuring the transient effects of indirubin, we modified the EROD assay [29], which measures predominantly CYP1A1 enzyme activity [35], in order to determine the effects of indirubin on CYP induction. Confluent MCF-7 cultures were exposed to 0.1% (v/v) DMSO, 10 nM indirubin, or 10 nM TCDD for various time periods up to 24 hr, and were then assayed for EROD activity. As seen in Fig. 4, at 3 hr post-exposure, induction of EROD activity by indirubin and by TCDD was similar: 3.8- and 4.1-fold over the initial level, respectively. Induction of EROD activity by TCDD, which is not appreciably metabolized in the cultures, persisted and was maximal at 20–24 hr after exposure. This induction by TCDD was about 20-fold higher than the initial EROD activity. However, EROD induction by indirubin was maximal

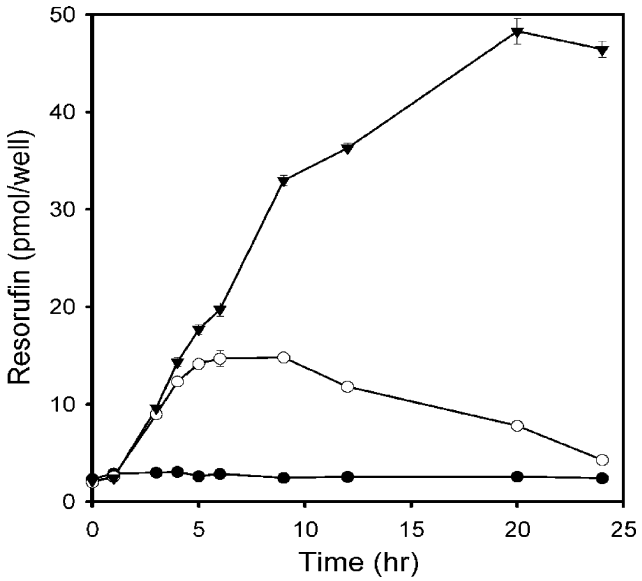

Fig. 4. Time course of the effects of indirubin and TCDD on CYP activity. MCF-7 cultures were exposed to 10 nM TCDD (▼), 10 nM indirubin (○), or 0.1% (v/v) DMSO (●) over a 24-hr period. At the indicated times, cultures were assayed for EROD activity as described in Section 2. Values are expressed as pmol resorufin formation per well and represent the mean ± SEM of determinations from four cultures of cells.

between 6 and 9 hr post-exposure, reaching 6.3-fold over the initial level. The activity declined thereafter, returning to a near-basal level by 24 hr.

To examine the concentration–response for induction of EROD activity by indirubin, we exposed confluent MCF-7 cells to varying concentrations of indirubin and TCDD for 4 hr. The results, shown in Fig. 5, indicate that induction of EROD activity was maximal at 10 nM indirubin or 10 nM TCDD. The EC_{50} values for stimulation of EROD activity were 1.26 nM and 0.32 nM for indirubin and TCDD, respectively.

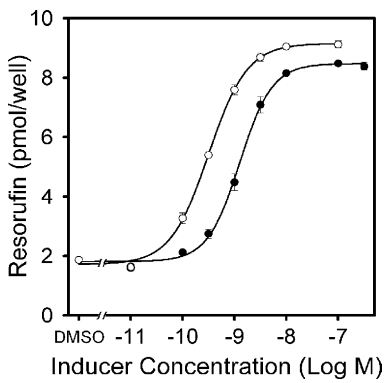

Fig. 5. Concentration–response curves for the effects of indirubin and TCDD on CYP activity as measured by EROD activity. MCF-7 cultures were exposed to 0.1% DMSO, or to the indicated concentrations of indirubin (●) or TCDD (○). After 4 hr, cultures were assayed for EROD activity as described in Section 2. Values are expressed as pmol resorufin formation per well and represent the mean ± SEM of determinations from four cultures of cells.

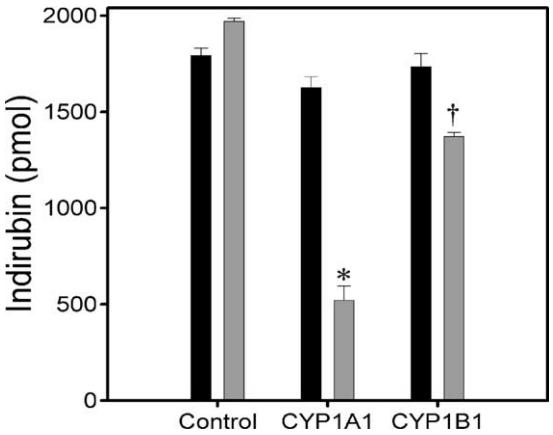

Fig. 6. Metabolism of indirubin by CYP1A1 and CYP1B1. Microsomes containing cDNA-expressed CYP1A1 or CYP1B1 and control microsomes were incubated with 10 μM indirubin at 37° for 30 min, either with (gray bars) or without (filled bars) NADPH, extracted with ethyl acetate, and analyzed for indirubin levels by HPLC as described in Section 2. Indirubin levels represent the mean ± SEM of determinations from three enzyme reactions. Symbols indicate significantly different from enzyme reactions without NADPH ($^{*}P < 0.001$, $^{\dagger}P < 0.05$).

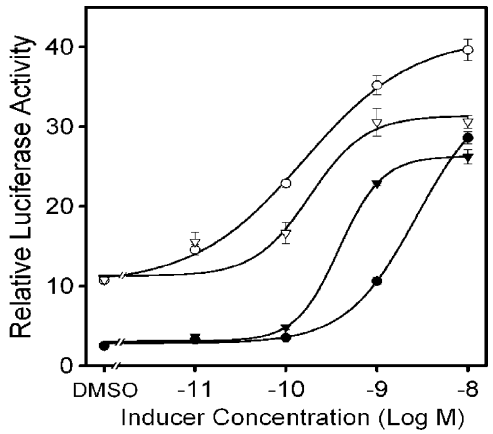

Fig. 7. Concentration–response curves for the effects of indirubin and TCDD, with or without ellipticine, on CYP1B1 promoter activity, as measured in a dual luciferase reporter assay. MCF-7 cultures were transiently transfected with p1B1Fluc and pRL-CMV as described in Section 2. Twelve hours later they were exposed to DMSO or to the indicated concentrations of indirubin either without (●) or with (○) 100 nM ellipticine, or to the indicated concentrations of TCDD either without (▼) or with (▽) ellipticine. After 12 hr, cultures were assayed for luciferase activities as described in Section 2, and light units from firefly luciferase activity were normalized to those from *Renilla* luciferase activity. Values represent the mean ± SEM of determinations from three cultures of cells.

3.4. Metabolism of indirubin by cDNA-expressed CYP1A1 and CYP1B1

Since indirubin induces CYP1A1 and CYP1B1, but only transiently, we investigated whether these enzymes are responsible for the metabolism of indirubin. Indirubin (10 μM) was incubated with *Sf9* microsomes containing either human CYP1A1 or CYP1B1, or with control *Sf9* microsomes (Gentest), to determine whether an NADPH-dependent decrease in indirubin concentration, indicative of P450-catalyzed metabolism, could be detected upon analysis by HPLC. After a 30-min incubation, we observed a 68% loss of indirubin in microsomal incubations with expressed human CYP1A1, and a 30% loss with incubations with expressed human CYP1B1, as compared to control reactions without NADPH (Fig. 6). Indirubin concentrations were not diminished in the control *Sf9* microsomal reactions with NADPH present.

We considered the possibility that the observed loss of indirubin in these microsomal reactions was due to metabolism catalyzed by human OR, which was co-expressed with both CYP1A1 and CYP1B1. At OR levels equivalent to those present in the CYP1A1 enzyme reaction shown in Fig. 6, we incubated 10 μM indirubin either with insect control microsomes, with or without added hOR (Gentest) or with microsomes exogenously expressing rat P450 OR (Gentest). No reduction in indirubin concentration was observed in these incubations with only OR and NADPH (data not shown).

3.5. Effect of indirubin on CYP1B1 promoter activity

Since CYP1A1 and, to a lesser extent, CYP1B1 metabolize indirubin and may have reduced the effective concentration of indirubin in EC_{50} determinations by the EROD assay, we examined the effect of ellipticine, a type II P450 inhibitor [32], on EC_{50} determinations in MCF-7 cells transiently transfected with a CYP1B1 promoter luciferase construct, p1B1Fluc. After transfection, MCF-7 cells were exposed to DMSO or to increasing concentrations of indirubin or TCDD, with or without ellipticine, for 12 hr, followed by assay for luciferase activity (Fig. 7). Levels of luciferase activity in the absence of an exogenous AhR ligand increased over 4-fold in response to treatment with ellipticine. The EC_{50} values for stimulation of luciferase activity by indirubin, with and without ellipticine, were 0.16 and 2.73 nM, respectively. The EC_{50} values for stimulation of luciferase activity by TCDD, with and without ellipticine, were 0.18 and 0.38 nM, respectively. MCF-7 cells were also transfected with a truncated luciferase construct, p1B1trFluc [25], in which the region containing five characterized DREs [31] was deleted from the CYP1B1 promoter. When cells transfected with p1B1trFluc were exposed to indirubin with or without ellipticine, no induction of luciferase activity was observed (data not shown), indicating that indirubin-induced transcriptional effects are AhR-mediated, and that ellipticine does not produce luminescence.

4. Discussion

Numerous studies, notably those employing cells derived from AhR-null mice [11,12], indicate roles of the AhR in cell-cycle control and in the regulation of cell

proliferation. Processes involving the AhR observed in the absence of exogenous ligands have led to the hypothesis that an endogenous ligand(s) of the AhR exists, and considerable effort has been dedicated to the identification of such ligands that may mediate such processes [18]. Some of the endogenous compounds identified to date that have binding affinity for the AhR include bilirubin [36]; lumichrome, a metabolite of riboflavin [37]; the arachidonic acid metabolite, lipoxin A_4 [38]; and the tryptophan metabolites, tryptamine and indole acetic acid [39,40]. Although these compounds are endogenous, they are relatively weak AhR agonists, and may not be present in the cell at sufficient concentrations to produce significant activation of the AhR. The tryptophan photoproduct, 6-formylindolo[3,2-*b*]carbazole, induces CYP1A1 at picomolar concentrations [41,42], but it may not be endogenous to the cell. Recently, a novel ligand for the AhR, 2-(1′*H*-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester, was isolated from porcine lung [43]. This compound, which was a potent AhR agonist in a DRE-driven reporter gene assay, could be synthesized *in vivo* by a condensation reaction of tryptophan with cysteine.

The results reported here suggest that indirubin induces its own metabolism in MCF-7 breast cancer cells through induction of CYP1A1 and CYP1B1. Evidence for negative feedback regulation of *CYP1A1* gene transcription mediated by the activity of CYP1A1 has been reported in other studies. Elevated basal CYP1A1 mRNA levels were observed in murine cell lines that lacked functional CYP1A1 protein [42,44]. Introduction of murine cyp1a1 or human CYP1A2 cDNA expression plasmids restored repression of cyp1a1 or NAD(P)H:menadione oxidoreductase, another member of the AhR gene battery [44]. Likewise, treatment of mouse hepatoma wild-type cells with the P450 inhibitor, ellipticine [32], resulted in elevated CYP1A1 mRNA expression [42] and elevated AhR-dependent reporter gene activity [45]. These studies suggest that cells lacking CYP1A1 activity accumulate an endogenous ligand of the AhR that is also a CYP1A1 substrate. Our results, which show the induction of CYP1A1 by indirubin, and the subsequent metabolism of this compound by CYP1A1, indicate that indirubin, in this respect, resembles an endogenous ligand of the AhR.

We found, as did Chang and Puga [45] by use of a similar assay, that treatment with ellipticine, without the addition of an inducer, increased basal activity in a CYP1B1 promoter-driven luciferase assay (Fig. 7), suggesting that inhibition of CYP1A1 allowed accumulation of an endogenous ligand, as ellipticine is not an AhR agonist [46]. In this same assay, the EC_{50} values for TCDD and indirubin were measured, both with and without ellipticine. When CYP1A1 was inhibited with 100 nM ellipticine, the apparent EC_{50} value for indirubin was reduced by 17-fold, to a level comparable to that for TCDD. The apparent EC_{50} value for TCDD, which is not readily metabolized by CYP1A1, was only reduced 2-fold with ellipticine treatment. In the EROD assay, in which inhibition of CYP1A1 activity is not feasible, the EC_{50} value determined with indirubin treatment was 4-fold higher than that determined with TCDD treatment, indicating that an accurate EC_{50} value for indirubin cannot be obtained under conditions where the concentration of indirubin is effectively reduced by CYP1A1 metabolism.

In MCF-7 cells, we found that induced CYP1A1 and CYP1B1 mRNA levels peaked at 3-hr exposure to indirubin and were absent by 12 hr. As measured by the EROD assay, induced CYP activity peaked at 6–8 hr and was nearly absent by 24 hr. This time course for CYP1A1 induction by indirubin is remarkably similar to those observed for bilirubin [36], lipoxin A4 [38], and 6-formylindolo[3,2-*b*]carbazole [42], other putative endogenous ligands of the AhR. Cell culture conditions that are hypothesized to induce an endogenous ligand, such as hydrodynamic shear, which induces EROD activity [15], or cell suspension, which induces cyp1a1 mRNA [14], also follow this time course of induction. In mouse hepatocytes, cyp1a1 mRNA was induced by medium change alone, peaking at approximately 6–9 hr and undetectable by 24 hr [47]. Photo-oxidized or autoclaved tryptophan, a component of the medium, induced CYP1A1 by an AhR-dependent mechanism [26], whereas induction of CYP1A1 by serum was found to occur by an AhR-independent pathway [48]. We likewise observed that fresh medium containing only the treatment vehicle, DMSO, increased EROD activities, and that these increases were due to a component of DMEM and followed a time course similar to that for indirubin-treated cells (data not shown). In order to avoid the confounding effects of media on CYP induction, we adopted a procedure similar to that of Kress and Greenlee [49] in our time courses and EC_{50} determinations, by adding treatments directly to medium that had been exposed to cells for at least 2 days.

The E_2 metabolism assay, which can distinguish between the activities of CYP1A1 and CYP1B1, showed that indirubin treatment caused a 2.8-fold induction of CYP1A1 activity (Fig. 2B) over the activity of the DMSO control. CYP1B1 activity was increased only 1.5-fold over that of the DMSO control by indirubin treatment. This induction, although not statistically significant, was the same as that observed for TCDD treatment. One factor that may be responsible for the lower induction levels for 2- and 4-$MeOE_2$ formation compared with those for induction of EROD activity and CYP1A1 and CYP1B1 mRNA is that the values for 2- and 4-$MeOE_2$ formation represent average rates of formation over the initial 6-hr period of indirubin exposure, not the peak value. In addition, elevated levels of CYP1A1 and CYP1B1 may have been present in the DMSO controls under conditions in which treatments were added in fresh medium. Subsequent experiments indicated that addition of fresh medium to cultures caused small, transitory increases in EROD activity and the levels of the CYP1A1 and

CYP1B1 mRNAs (data not shown). The increases in CYP1A1 and CYP1B1 due to medium change may have resulted in higher control levels of 2- and 4-MeOE$_2$ formation, and thus lower levels of induction over control by indirubin.

Consistent with the E$_2$ metabolism results, the CYP1A1 mRNA level at 3 hr (Fig. 3) was increased over the level in the DMSO control to a greater extent than was the CYP1B1 mRNA level (9.6-fold compared with 3.2-fold, respectively). Also, the catalytic activity of CYP1B1 in metabolizing indirubin, as seen in Fig. 6, was lower than that of CYP1A1. CYP1A1 and CYP1B1 have distinct but overlapping substrate specificities and can produce, as with E$_2$, different metabolites. Several hydroxy metabolites of the tryptophan photoproduct, 6-formylindolo[3,2-*b*]carbazole, produced in rat liver microsomal preparations have recently been identified [50]. Although it is tempting to speculate that CYP1A1 and CYP1B1 activities produce hydroxy metabolites of indirubin, the characterization of the possible metabolites awaits further study.

Our results agree with those of Adachi *et al.* [20], who found that indirubin is a very potent AhR ligand which is active at nanomolar concentrations. Indirubin may thus have the capacity to regulate cellular proliferation by interaction with the retinoblastoma protein, causing cell-cycle arrest [4,5] or by induction of the cell-cycle inhibitor, p27^{Kip1} [6]. Aside from its potential role as a ligand of the AhR, indirubin was shown to be the active ingredient of a Chinese antileukemia medicine [51] and to inhibit proliferation of several cell lines, including MCF-7, at micromolar concentrations [51,52]. It is worth noting that several derivatives of indirubin were shown to directly inhibit the cell-cycle regulators, cyclin dependent kinases and glycogen synthase kinase-3β, at 5–100 nM concentrations [51,53]. It is possible that the products of phase I and phase II metabolism of indirubin, which have not yet been identified, may contribute to its pharmacological and antiproliferative properties.

The preponderance of data suggests that the most likely candidates for high-affinity endogenous ligands of the AhR are, like indirubin, derivatives of indole [20,41–43], and that the ligands are metabolized by the enzymes that they induce. An argument against indirubin being an endogenous ligand of the AhR is that, even though the compound has been identified in mammalian urine and serum, its source has not been identified, and may in fact be dietary. The only characterized biochemical pathways leading to indirubin formation have been exclusively found in plants. However, it is known that products of indole metabolism catalyzed by mammalian CYPs will condense non-enzymatically to form indigo and indirubin. Since only nanomolar levels of indirubin are necessary to activate the AhR, and since its metabolism may be rapid, it is possible that biochemical pathways and the enzymes involved leading to indirubin formation in animals have yet to be identified.

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