Regulation of CYP1A1 by Indolo[3,2-b]carbazole in Murine Hepatoma Cells*

(Received for publication, April 17, 1995, and in revised form, July 11, 1995)

Yue-Hwa Chen†, Jacques Ribi‡, Pramod Srivastava†, James Bartholomew§, Micheal Denison¶, and Leonard Bjeldanes†

From the Department of Nutritional Sciences and §Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720 and the ¶Department of Environmental Toxicology, University of California, Davis, California 95616

To determine the basis for unexpected differences in CYP1A1 inducing potencies and efficacies for the diet-derived indole derivative, indolo[3,2-b]carbazole (ICZ) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), we conducted a systematic analysis of events involved in the induced expression of CYP1A1 in murine hepatoma-derived cell lines (Hepa-1). In contrast to the effects of TCDD, induction kinetics and CYP1A1 mRNA half-life were dependent on ICZ concentration, and the response from low doses of inducer was transient due to rapid clearance of ICZ. TCDD and ICZ produced the same maximum response (i.e. equal efficacies) from a TCDD-responsive CAT reporter construct in Hepa-1 cells. When measured by the immediate responses associated with CYP1A1 expression, including cellular uptake of inducer, receptor transformation and binding to DRE (gel mobility shift assay), initiation of transcription (nuclear run-on assay), and short-term accumulation of mRNA (Northern blot assay), ICZ also exhibited an efficacy equal to that of TCDD and a potency that corresponds to its receptor affinity. ICZ is a potent and selective noncompetitive inhibitor of ethoxyresorufin O-deethylase activity (Kᵢ = 1.5 nm). Taken together these results indicate that ICZ is a bifunctional modulator of CYP1A1 expression with intrinsic efficacy equal to that of TCDD.

Indolo[3,2-b]carbazole (ICZ) is a compound of dietary origin present in the gastrointestinal tract of rodents and humans. ICZ is produced in vivo and in vitro as one of the acid-catalyzed reaction products of non-nutritive indoles such as indole-3-carbinol and glucobrassicin that are present in cabbage and Brussels sprouts and other plants of the Brassica genus (1–4). ICZ is also produced, presumably from the nutritive indole, tryptophan, as a metabolic product of intestinal bacteria (5).

ICZ is similar in several respects to the potent environmental pollutant, TCDD. Both compounds have immunosuppressive activity in murine fetal thymus organ culture and both substances exhibit potent antiestrogenic activities including inhibition of estrogen-dependent growth of cultured breast tumor cells (6, 7). Additionally, both ICZ and TCDD induce CYP1A1 activity in animals and in cultured cells (1). CYP1A1 is a phase I enzyme involved in the metabolism of many drugs and carcinogens. CYP1A1 is also the enzyme thought to be primarily responsible for the inactivation of estradiol in breast tumor cells (8).

Perhaps key to these similarities in activities is the fact that ICZ and TCDD are nearly isosteric and both compounds are potent Ah receptor agonists (9, 10). The Ah receptor is a widely occurring, ligand-activated transcription factor that mediates the activation of CYP1A1, CYP1A2, glutathione S-transferase Ya, and quinone reductase genes. Binding to this receptor is thought also to be responsible for most of the toxic effects of TCDD, including tumor promotion, teratogenesis, and lethality with wasting, that appear to result mechanistically from effects beyond simple induction of xenobiotic metabolizing enzymes (11).

Current knowledge of the Ah receptor-mediated signaling pathway derives primarily from studies of CYP1A1 induction. The process involves ligand binding to a cytosolic Ah receptor/ Hsp90 complex which produces a conformational change that results in translocation of the complex to the nucleus where the receptor combines with the Ah receptor nuclear transporter protein. The heterodimer binds to receptive DNA elements (9–11) located in the enhancer region of the CYP1A1 gene (12–14). By mechanisms yet to be determined, this process leads to an increase in transcription rate.

We are interested in the mechanism of action of ICZ as a model natural ligand for the Ah receptor. Although ICZ exhibits high affinity for the Ah receptor, it is halogen-free with low lipophilicity. ICZ, therefore, is less likely to accumulate and persist in cells than is TCDD and may exhibit certain properties, including the long-term effects, that are quite different from those of TCDD. In previous studies we noted that (a) TCDD is 10³–10⁶ times as active as ICZ in the induction of CYP1A1-dependent enzyme activity in Hepa-1 cells, a difference that is at least 2 orders of magnitude larger than the difference in binding affinities for the Ah receptor, and (b) the maximal enzyme activity induced by ICZ in the cells is only about 60% the maximal activity induced by TCDD (1). In the present study we compared further the CYP1A1 regulatory activities of ICZ and TCDD in the murine hepatoma cell line in an effort to explain these differences in their activities and to gain further understanding of the regulation of this important gene.

EXPERIMENTAL PROCEDURES

Materials

ICZ was prepared by K. Grose (University of California, Berkeley, CA) according to the procedure of Robinson (15). TCDD samples were obtained from B. Ames (University of California, Berkeley, CA) and S.
used as a probe to detect the CYP1A1 mRNA band at 2.9 kilobases, was kindly provided by D. Nebert (University of Cincinnati, OH). The human β-actin cDNA probe (Clontech Laboratories, Palo Alto), gave a mRNA band at 2.1 kilobases. Both probes were labeled with \( ^{32}P \)JUMP by random primer labeling kit (Stratagene) and were then purified by the Elutip kit (Schleicher & Schuell). The nylon membrane blot was prehybridized, hybridized (0.5 M sodium phosphate buffer, 0.1 M EDTA, 7% SDS, 0.7% bovine serum albumin, and 65 °C), and washed (65 °C, 0.5 M sodium phosphate buffer, 1 M EDTA, 5% and 1% SDS, and 0.5% bovine serum albumin) as described (19). For analysis of β-actin mRNA, blots were stripped (20) (0.1 × SSC, 0.1% SDS, 95 °C) and rehybridized with the β-actin probe. mRNA was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Ligand-induced Expression of a DRE-driven Reporter Gene

Stable Transfection of Hepa-1 Cells with DRE/CAT Reporter—Hepa-1 cells were cotransfected with plasmids pcDNA 3 as a transfection marker that confers resistance to the antibiotic derivative G418 (geneticin), and with the pMCat 5.9 plasmid, which is a chimeric CAT reporter under the control of the Cyp1a-1 enhancer/murine mammary tumor virus promoter. The pMCat plasmid was a kind gift from J. P. Whitlock, J. r. (Stanford University, Palo Alto, CA). Transfection was done by the calcium phosphate coprecipitation method followed by a glycerol shock. Each 100-mm culture dish at 1:15 confluence was transfected with 30 μg of pcDNA 3 and 30 μg of pMCat 5.9. After 2 days of growth in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, cells were split 1:15 in the same medium containing G418 (500 μg/ml). Cells were re-fed every 4 days with the same selection medium for 2 weeks and individual colonies were subcloned and tested for inducible CAT activity.

Assay of CAT Activity—We assayed chloramphenicol acetyltransferase activity by the phase extraction assay (30). Near confluent plates (0.8–1.0 × 10^7 cells) were treated with various concentrations of inducer for up to 20 h to induce CAT expression. Cells were harvested by trypsination, resuspended in culture medium, and centrifuged. Cell pellets were resuspended in hypotonic Tris buffer and incubated at room temperature for 5 min and the supernatant was centrifuged. The cell pellets were resuspended in Tris buffer containing 0.1% Triton X-100, incubated at room temperature for 5 min, and the lysates were centrifuged to remove nuclei. Aliquots of cell lysates were incubated at 65 °C for 10 min to inactivate inhibitors. Substrates ([H]chloramphenicol and butyryl-CoA) were added and the reaction mixture was incubated at 37 °C for 30 min. The reactions were stopped by the addition of tetrathymylenatedecane, 10% trichloroacetic acid, and 10% butyryl-CoA before being added to the assay medium. The production of [3H]-butylated chloramphenicol was counted for radioactivity.

Gel Retardation Assay

Cells were incubated with inducers for 1 h and nuclear extracts were prepared from MeSO-, TCDD-, or ICZ-treated Hepa-1 cells as described previously (23, 24). A complementary pair of synthetic oligonucleotides containing the sequence 5'-GATCTGGCTCTTCTACTCCGAACCTTGGGGAAGGACCC-3' (corresponding to the Ah receptor (AhR) binding site of DRE and designated as the wild-type DRE oligonucleotide) and 5'-GATCTGGCTCTTCTACTCCGAACCTTGGGGAAGGACCC-3' (identical to the wild-type DRE oligonucleotide but containing a single substitution (underlined) within the DRE core consensus sequence which eliminates binding of the transformed TCDD-AhR complex and designated as the mutant DRE (mDRE) oligonucleotide), were synthesized, purified, annealed, and radiolabeled with \( ^{32}P \)JUMP as described (25).

Gel retardation analysis of MeSO-, TCDD-, and ICZ-treated Hepa-1 nuclear extracts was carried out as described previously (23, 24). To determine the amount of protein-DNA complex formed, the specific radiolabeled band was excised from the dried polyacrylamide gel and quantified by liquid scintillation. The amount of \( ^{32}P \)-labeled DRE specifically bound in the ligand-inducible complex was estimated by measuring the amount of radiolabeled DRE isolated from the ligand-treated sample lane, and subtracting the amount of radiolabeled DRE present in the same position in a non-ligand-treated sample lane. The difference in radioactivity between these samples represents the ligand-inducible specific binding of \( ^{32}P \)-DRE and is expressed as the amount of ligand-AhR-DRE complex formed.

Nuclear Run-on Assay

Nuclei were isolated at various times up to 8 h following ICZ (1 μM) or TCDD (1 nm) treatment and incubated with \( ^{32}P \)JUMP and unlabeled

---

**Structure 1.**

| a | indol(3,2-b)carbazole; b, indole-3-carbinol; c, glucobrassicin. | Safe (Texas A&M University, College Station, TX). Ethoxyresorufin was purchased from ICN Biochemical Co., and resorufin was from Aldrich, [\( ^{32}P \)]JCTP (3000 Ci/mmol) was from Amersham and [\( ^{32}P \)]ATP (6,000 Ci/mmol) was from DuPont NEN. All cell culture and molecular biological grade chemicals were purchased from Boehringer Mannheim, Fisher, Sigma, or from Life Technologies, Inc. HPLC grade Me2SO was from Sigma. Sodium phenobarbital was from Mallinckrodt Chemical Works. |
ATP, CTP, and GTP as described (26). The radiolabeled RNA transcripts were isolated and hybridized to an excess of denatured Cyp1a1 plasmid DNA immobilized on a nitrocellulose filter. Blots were quantified by PhosphorImager and data were normalized by comparison to the transcriptional level of β-actin.

mRNA Stability

Confluent Hepa-1 cells were preincubated with ICZ or TCDD for 8 h and then treated with actinomycin D, at a final concentration of 2 μg/ml. Our preliminary experiment was consistent with results of others (21) and indicated that this concentration of actinomycin D fully blocked the induction of Cyp1a1 transcription in response to ICZ or TCDD. Total RNA was then isolated at different time points and quantified after gel electrophoresis and Northern hybridization. The half-life of the mRNA was calculated by extrapolation from the logarithmically transformed best fit line (22). The data were plotted on a semi-logarithmic scale, as percentage of CYP1A1 mRNA remaining versus time.

In Vitro Inhibition of CYP1A1 (EROD) and CYP2B (Pentoxyresorufin O-Dealkylase) Activities by ICZ

The microsomal EROD and pentoxyresorufin O-dealkylase activities were determined by a modification of published methods (16, 28). Microsomal protein (100–200 μg) was added to 1.4 ml of 0.1 M potassium phosphate buffer (pH 7.8), followed by the step-wise addition of 15 μl of ethoxyresorufin or pentoxyresorufin (0.52 μM) in ethanol, 10 μl of ICZ in Me2SO, and 7.5 μl of 50 μM NADPH in buffer. The rate of production of resorufin was then determined by fluorescence analysis at 30°C. The protein content was determined by the Bio-Rad protein assay, with bovine serum albumin as a standard. Activities are expressed as resorufin pmol/min/mg of protein.

To determine the EROD inhibitory effect of ICZ over time, various concentrations of ICZ were preincubated with the induced microsomes, and the NADPH and the substrate were added at the end of incubation for enzyme analysis. IC50 values (ICZ concentration giving 50% inhibition of EROD activity) were estimated from the inhibition curves, and the inhibition constant (Ki) was determined by Dixon plot (29).

HPLC Analysis of ICZ Disappearance

ICZ (10 nm) was incubated with the induced microsome/NADPH metabolic mixture and aliquots were taken at intervals up to 30 min for ethyl acetate extraction and HPLC analysis of ICZ by procedures described previously (5).

RESULTS

Concentration-dependent Induction of CYP1A1 Enzymatic Activity and mRNA, and Activation of a CAT Reporter by ICZ and TCDD—Fig. 1 illustrates the concentration-response curves for EROD activity induced by ICZ and TCDD after either 24 or 48 h treatment of wild-type Hepa-1 cells. There was no significant shift in the curves between 24- and 48-h treatments. The maximal EROD induction response after 48 h was about 60% of the maximum induced by TCDD and the EC50 values differed by nearly 4 orders of magnitude. EROD activity was not detectable in the AhR nuclear translocator-deficient mutant cells 24 h after treatment with a range of doses of ICZ (data not shown).

Fig. 2 shows the results of Northern blot analyses of the CYP1A1 mRNA levels relative to β-actin mRNA in Hepa-1 cells expressed as percent of maximum induction after 4- and 24-h treatment with a range of concentrations of TCDD and ICZ. Whereas there was no significant shift in the TCDD curve, there was a clear shift to the left of the 4-h curve for ICZ compared to the 24-h curve. The difference in EC50 values at 4 h was about 2 orders of magnitude and the maximum level of induction by ICZ after 4 h incubation was similar to the maximum level induced by TCDD.

Results of an experiment to examine the TCDD and ICZ concentration-dependent activation of chloramphenicol acetyltransferase in Hepa-1 cells stably transfected with a TCDD-responsive bacterial CAT reporter gene are shown in Fig. 3. The EC50 values for CAT reporter induction by 19 h treatment with TCDD and ICZ are similar to the 24-h EC50 values for EROD and CYP1A1 mRNA induction by these compounds and their potencies differ by over 4 orders of magnitude in all three assays. In contrast to the results of EROD induction experiments, however, the maximal levels of CAT activation were similar for the two inducers.

Kinetics of Induction of EROD Activity and CYP1A1 mRNA by ICZ and TCDD—Studies of the kinetics of EROD induction by high (1.35 μM) and low (67.5 nM) concentrations of ICZ indicated that maximal induction occurred at 8 h for the lower concentration of ICZ and at 16 h for the higher concentration of ICZ (Fig. 4). The EROD activity induced by a low concentration of ICZ was transient, falling to 40% of the maximal level by 48 h, and activity induced by a high dose of ICZ persisted for at least 48 h.

Results of similar studies of steady-state CYP1A1 mRNA
levels indicated that the maximal induction of message occurred around 4 h of treatment with the lower concentration of ICZ and around 8 h in response to the higher concentration of ICZ. Consistent with the kinetics for induction of enzyme activity, the level of CYP1A1 mRNA induced by a low concentration of ICZ was also transient and fell to about 20% of the maximal level by 16 h (data not shown).

Effect of Removal or Addition of ICZ in the Medium on Expression of CYP1A1—To examine whether Hepa-1 cells become insensitive to ICZ, they were preincubated with 270 nM ICZ for 24 h and then ICZ was removed or was added at the times indicated; group I, no change in medium for 72 h; group II, additional ICZ was added at 48 h; group III, additional ICZ was added at 24 h; group IV, the medium was replaced with ICZ-free medium at 24 h. The cells were collected for analysis of EROD activity at the designated times. Symbols and bars represent mean values and the ranges of two individual determinations. RF, resorufin; DMSO, Me2SO.

**FIG. 5.** Effect of multiple additions of ICZ to the medium on EROD activity in Hepa-1 cells. The cells were incubated with 270 nM ICZ for 24 h and then ICZ was removed or was added at the times indicated; group I, no change in medium for 72 h; group II, additional ICZ was added at 48 h; group III, additional ICZ was added at 24 h; group IV, the medium was replaced with ICZ-free medium at 24 h. The cells were collected for analysis of EROD activity at the designated times. Symbols and bars represent mean values and the ranges of two individual determinations. RF, resorufin; DMSO, Me2SO.

Another 48 h, EROD activity dropped to about 15% of the 24 h level after a total of 72 h incubation in the same medium (group I). This decrease required only about 12 h after removal of ICZ from the medium (group IV). However, the activity increased for another 24 h when additional ICZ was added to the medium (groups II and III). Similar phenomena were observed in steady-state levels of CYP1A1 mRNA when cells were incubated with 67.5 nM ICZ (data not shown). In this case, CYP1A1 mRNA levels decreased by about 85% by 4 h following exposure of induced cells to ICZ-free medium. In contrast to ICZ-treated cells, subsequent exposure of TCDD-induced cells to TCDD-free medium did not result in a drop in EROD activity (data not shown).

**Cyp1a-1 Transcription Rate**—Comparisons by nuclear run-on assay of the Cyp1a-1 transcription rates induced by another 48 h. EROD activity dropped to about 15% of the 24 h level after a total of 72 h incubation in the same medium (group I). This decrease required only about 12 h after removal of ICZ from the medium (group IV). However, the activity increased for another 24 h when additional ICZ was added to the medium (groups II and III). Similar phenomena were observed in steady-state levels of CYP1A1 mRNA when cells were incubated with 67.5 nM ICZ (data not shown). In this case, CYP1A1 mRNA levels decreased by about 85% by 4 h following exposure of induced cells to ICZ-free medium. In contrast to ICZ-treated cells, subsequent exposure of TCDD-induced cells to TCDD-free medium did not result in a drop in EROD activity (data not shown).

Ligand-activated Nuclear Translocation and Binding of Ah Receptor to the DRE, in Vitro—To compare the abilities of TCDD and ICZ to transform the Ah receptor to a form that is translocated into the nucleus and binds to specific DNA elements, we conducted gel retardation assays using wild-type and mutant DREs. The results (Fig. 7) indicated nearly parallel dose-response curves with equal maximum responses, and EC50 values that differ by only about 2 orders of magnitude. We observed no ICZ-inducible protein DNA complex using 32P-labeled mutant DRE sequence (data not shown). This later result is consistent with the DNA binding specificity of the AhR and implies that this complex represents the ICZ-AhR-DRE complex.

**Cyp1a-1 Transcription Rate**—Comparisons by nuclear run-on assay of the Cyp1a-1 transcription rates induced by
high concentrations of TCDD and ICZ indicated that the same maximal rates were reached in less than 30 min following exposure of Hepa-1 cells to either inducer (data not shown).

**mRNA Stability**—The half-life of the CYP1A1 mRNA was determined by an actinomycin D chase experiment. There was no significant difference in the half-lives (4.2–4.8 h) of the CYP1A1 mRNA induced by low (10 pM) or high (1 nM) concentrations of TCDD or by a high (1.35 μM) concentration of ICZ. The half-life of the mRNA induced by a low concentration of ICZ (67.5 nM), however, was reduced to only about 1.7 h (Fig. 8).

**Inhibition of CYP Enzyme Activities by ICZ**—The inhibitory effects of ICZ on microsomal EROD activity are indicated in Fig. 9. The results indicated that ICZ is a potent, noncompetitive inhibitor of EROD activity in the microsomal system with \( K_i = 1.5 \text{ nM} \) computed from a Dixon plot. Similar plots were generated for ICZ inhibition of EROD activity in Hepa-1 cells (data not shown). No inhibitory effect of ICZ on microsomal pentoxyresorufin O-dealkylase activity was observed.

**Metabolic Clearance of ICZ**—Evidence of the rapid metabolic clearance of ICZ was provided by the results of two experiments. In the first experiment ICZ was preincubated for up to 10 min with hepatic microsomes from ICZ-treated rats or with TCDD-induced Hepa-1 cells. Fig. 10, a and b, indicate a rapid loss of ICZ's inhibitory effect on EROD activity under these conditions. In the second experiment, the disappearance of ICZ was measured directly by HPLC analysis of organic extracts from the incubation mixture. Fig. 10 c indicates that a greater than 90% loss of ICZ from the microsomal system occurred within 30 min. No loss of ICZ was seen during this period in control experiments in which ICZ was incubated in buffer either without microsomes or with microsomes previously heated at 100 °C for 1 min to inactivate them. The results of these experiments indicate that ICZ is rapidly metabolized to inactive substances.

**DISCUSSION**

The purpose of this study was to examine the CYP1A1-inducing effects of ICZ as a model natural ligand for the Ah receptor. We have shown that mutant cells deficient in Ah receptor function do not possess detectable EROD activity after ICZ treatment, which confirms the requirement for a competent Ah receptor/AhR nuclear translocator signal transduction system for CYP1A1 induction by ICZ, as for TCDD (31, 32). Our results, consistent with previous observations (1), demonstrated that TCDD and ICZ generated parallel concentration-response curves for induction of EROD activity with ICZ producing a lower maximal response than TCDD. Concentration-response curves for induction of the TCDD-responsive CAT reporter and for CYP1A1 mRNA were also parallel for the two inducers, but in these cases maximal responses similar to that of TCDD were produced by ICZ (Figs. 1–3). Simultaneous treatment of cells with maximally inducing concentrations of both inducers produced no greater response than either inducer alone. This evidence indicates that ICZ and TCDD function by the same mechanism in the induction of CYP1A1 in the Hepa-1 cells and that while ICZ is a less potent inducer than TCDD, it has the same efficacy as an inducer.

In contrast to the effects of TCDD, however, the kinetics of
CYP1A1 induction by ICZ were dependent on the dose of inducer. Maximum induction of CYP1A1 mRNA occurred after 8 h of exposure to the higher concentration of ICZ, and after only 4 h incubation with the lower dose of ICZ. Maximum induction of CYP1A1 mRNA occurs after 8 h of exposure to TCDD (37,38) and in this case the kinetics are reported to be independent of dose of TCDD in the Hepa-1 cells (26).

Also in contrast to the response from TCDD, CYP1A1 induction by a low concentration of ICZ (67.5 nM) was transient. This transient effect also has been observed in rainbow trout treated with indole-3-carbinol, a precursor of ICZ (39), and in rodent liver tumor cell lines exposed to acryl hydrocarbons (40, 41). This transient induction by ICZ in the Hepa-1 cells is most likely due to the clearance of ICZ from the medium. When induced cells were incubated in ICZ-free medium, the EROD activity and CYP1A1 mRNA level dropped by about 85% in 12 and 4 h, respectively. The induction was recovered when ICZ was reintroduced into the medium, indicating that the cells had not become insensitive to the inducer and that the signal transduction system had not been down-regulated following exposure to ICZ. In contrast, following removal of TCDD from the medium, the induced EROD activity remained unchanged for at least another 48 h. The persistent effect of TCDD has been reported by several investigators (38, 40, 42) and is likely due to its high resistance to metabolic degradation.

Since the steady-state level of mRNA is affected by rate of transcription and rate of mRNA degradation, we conducted a series of experiments to compare the effects of ICZ and TCDD on individual components of the Ah receptor-mediated signal transduction pathway. Comparisons of ICZ- and TCDD-induced transformation of the Ah receptor, its translocation to the nucleus, and its binding to DNA, in vitro, indicated that there was an approximate 100-fold difference in potencies for the two compounds and that they produced the same maximum level of transformation and binding to DNA. This difference in potencies, which was observed after 1 h exposure to inducer, corresponds to the difference in Ah receptor binding affinities that we have determined for the compounds. The similarities in maximum levels of Ah receptor transformation, induced mRNA levels, and maximum transcription rate (based on our nuclear run-on experiments), indicate further that while the two compounds have different potencies, they are equally effective as CYP1A1 inducers. This is in contrast to 3-methylcholanthrene, benzoanthracene, and certain chlorinated hydrocarbons that have intrinsic inducing efficacies that do not correspond to their high affinities for the Ah receptor (40, 43, 44).

To compare the effect of ICZ on the degradation rate of the CYP1A1 mRNA we also performed actinomycin D chase experiments. Our results showed that induction with either low (10 pM) or high concentrations (100 pM) of TCDD or a high concentration of ICZ (1.35 mM) produced CYP1A1 mRNA with a half-life of about 4.5 h. This value is consistent with a 3-h half-life computed previously for CYP1A1 mRNA in Hepa-1 cells and not directly measured (32), but it is considerably shorter than the 14-h half-life previously measured in β-naphthoflavone-induced rabbit hepatocytes (33). Treatment of cells with a low concentration of ICZ (67.5 nM) produced CYP1A1 mRNA with a half-life of only about 1.7 h. This dependence of the mRNA half-life on inducer concentration has not been reported previously for Hepa-1 cells. Post-transcriptional regulation of CYP1A1, however, has been reported for 3-methylcholanthrene- and TCDD-induced rat hepatocytes, and in TCDD-induced mouse livers based on the difference between the magnitude of the increase in steady-state mRNA accumulation and the rate of transcription (34–36). Post-transcriptional mechanisms also contribute to the regulation of the CYP1A2 gene as indicated by results of studies of TCDD- and 3-methylcholanthrene-induced rat hepatocytes and livers (35, 36), and in the β-naphthoflavone-induced rabbit hepatocytes (33). Our results are consistent with a direct role of the inducer in stabilization of message.
ICZ is not only an inducer of the Cyp1a-1 gene, but also a potent and selective inhibitor of CYP1A1 enzyme (EROD) activity. With an inhibition constant of 1.5 nM for EROD activity, ICZ is the most potent of the various synthetic (e.g. α-naphthoflavone and 1-ethyllynylpyrene) and naturally occurring (e.g. ellipticine, flavonoids, and coumarins) inhibitors for which inhibition data are available (45–48). Because ICZ is similar in chemical structure to ellipticine and both compounds are non-competitive inhibitors, it is likely that ICZ functions by the mechanism suggested for ellipticine, that is, by association with heme and displacement of oxygen from the active site (49).

ICZ, however, does not exhibit the high degree of cytotoxicity that is characteristic of the ellipticines. A selectivity in enzyme inhibition for ICZ is indicated by its lack of effect against phenobarbital-induced CYP2B1/2B2 (pentoxysorufin O-dealkylase) activity. The lower maximal induction of EROD activity induced by ICZ and the suppressive effect of high doses of ICZ on TCDD-induced EROD activity that we observed can be attributed to the inhibitory effect of ICZ.

Our observation of the potent inhibitory effect of ICZ against EROD activity provided an indirect method of evaluating ICZ cellular uptake and clearance. The rapid onset and subsequent loss of this inhibition and the similar kinetics in incubations with both microsomes and cells are consistent with a rapid cellular uptake and clearance of ICZ by metabolism to inactive substances. Rapid uptake of ICZ is further indicated by our results showing the potent and rapid effect of ICZ on Ah receptor nuclear translocation and DNA binding and by the rapid induction of transcription indicated by the results of the nuclear run-on assay. Direct evidence for the rapid clearance of ICZ was provided by the results of HPLC analyses of the microsomal incubation mixture. These observations indicate that the decreased potency for CYP1A1 induction by ICZ compared to TCDD is not due to a significantly decreased rate of ICZ uptake but is due to rapid clearance.

In light of our results, a recent report by Kieley et al. (50) suggests that the inducing effects of indolocarbazoles may be highly dependent on variations in inducer structure, cell-type, or enhancer configuration. In contrast to our findings on the response to ICZ in Hepa-1 cells of the chloramphenicol acetyltransferase reporter gene controlled by the full Cyp1a-1 enhancer, the N,N′-dimethylated derivative of ICZ, 5,11-dimethylindolo[3,2-b]carbazole is reported to be equipotent with TCDD in these cells as an inducer of transcription from a CAT reporter gene controlled by a simple, single DRE-containing enhancer. In addition, ICZ and 5,11-dimethylindolo[3,2-b]carbazole are reported to be equipotent with TCDD as inducers of the latter reporter in human hepatoma cells (HepG2). These increased potencies of the indolocarbazoles relative to TCDD could result from decreased metabolic clearance and/or an increased sensitivity to the indolocarbazoles of the Ah receptor-mediated signal transduction pathway.

Taken together our results indicate that ICZ is a bifunctional modulator of CYP1A1 expression in murine Hepa-1 cells. ICZ and TCDD function by the same mechanism and with equal efficacy in the induction of CYP1A1. The decreased potency of ICZ in comparison to its affinity for the Ah receptor may be attributed to rapid clearance of the inducer from these cells. Clearance of inducer also appears to be responsible for the transient ICZ-induced expression of CYP1A1, an effect that is augmented by decreased stability of CYP1A1 mRNA in the absence of the inducer. ICZ is also a selective, noncompetitive inhibitor of CYP1A1 enzyme activity, with a potency for enzyme inhibition that is greater by an order of magnitude than its maximum inducing potency. Thus, at concentrations below those necessary to produce gene activation, ICZ can inhibit CYP1A1-mediated enzyme activity. Whether these quantitative and qualitative effects of ICZ on CYP1A1 expression can be generalized to human systems requires further investigation.

Acknowledgments—We thank Dr. William Helferich for valuable discussions on the in vitro analysis of Ah receptor transformation. We also thank Dr. J. In-Young Park for technical assistance in the analysis of the combined effects of ICZ and TCDD on EROD activity, and Dr. Kathryn Tullis for assistance with gel retardation analysis.

REFERENCES

1. Bjeldanes, L. F., Kim, J.-Y., Grose, K. R., Bartholomew, J. C., and Bradfield, C. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9543–9547
2. Grose, K. R., and Bjeldanes, L. F. (1992) Chem. Res. Toxicol. 5, 188–193
3. deKruif, C. A., Marsman, J. W., Venekamp, J. C., Falke, H. E., Noordhoek, J., Blaauwboer, B. J., and Wartzelboer, H. M. (1991) Chem-Biol. Interact. 80, 303–315
4. McDanel, R., McLean, A. E. M., Hanley, A. B., Heaney, R. K., and Fenwick, G. R. (1988) Food and Chemical Toxicology 26, 59–70
5. Kwon, C.-S., Grose, K. R., Riby, J., Chen, Y.-H., and Bjeldanes, L. F. (1994) J. Agric. Food Chem. 42, 2536–2540
6. d’Argy, R., Bergman, J., and Dencker, L. (1989) Pharm. Toxicol. 64, 33–38
7. Liu, H., Wormald, M., Sane, S. H., and Bjeldanes, L. F. (1994) J. Natl. Cancer Inst. 86, 1758–1765
8. Spink, D. C., Eugster, H.-P., Lincoln, D. W., II, Schultz, J. D., Schultz, E. G., Johnson, J. A., Kaminiski, L. S., and Gierthy, J. F. (1982) Arch. Biochem. Biophys. 209, 342–348
9. Gillner, M., Bergman, J., Camilli, C., Fernstrom, B., and Gustafsson, J.-Å. (1985) Mol. Pharmacol. 28, 357–363
10. Gillner, M., Bergman, J., Camilli, C., Alexenrod-Meier, M., Fernstrom, B., and Gustafsson, J.-Å. (1993) Mol. Pharmacol. 44, 336–345
11. Landers, J. P., and Bunce, N. J. (1991) Biochem. J. 276, 237–287
12. Whitlock, J. P., Jr. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 251–277
13. Whitelaw, M. L., Gottlicher, M., Gustafsson, J.-Å., and Poellinger, L. (1993) EMBO J. 12, 4169–4179
14. Carver, L. A., Hogenesch, J. B., and Bradfield, C. A. (1994) Nucleic Acids Res. 22, 3038–3044

---

**Fig. 10. Kinetics of ICZ disappearance.** ICZ (4–10 nM) was incubated with ICZ-induced microsomes or TCDD-induced Hepa-1 cells for the times indicated, followed by either addition of the substrate for EROD assay of (a) microsomes and (b) cells, or extraction of aliquots of the microsomal mixture for analyses of ICZ by HPLC (c). Experiments were conducted twice with similar results.
Regulation of CYP1A1 by Indolo[3,2-b]carbazole 22555

15. Robinson, B. (1963) J. Chem. Soc. 3097–3099
16. Burke, M. D., and Mayer, R. T. (1974) Drug Metab. Dispos. 2, 583–588
17. Ausubel, F. M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1992) Current Protocols in Molecular Biology. Preparation and Analysis of RNA, pp. 4.2.5–4.2.8, John Wiley and Sons
18. Ausubel, F. M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1992) Current Protocols in Molecular Biology. Analysis of RNA by Northern Hybridization, pp. 4.9.1–4.9.8, John Wiley and Sons
19. Church, G. M., and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 1991–1995
20. Durrin, L. K., and Whitlock, J. P., Jr. (1989) Mol. Cell Biol. 9, 5733–5737
21. Harrold, S., Genovese, C., Kobrin, B., Morrison, S. L., and Milcarek, C. (1991) Anal. Biochem. 198, 19–29
22. Denison, M. S., Fisher, J. M., and Whitlock, J. P., Jr. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 2528–2532
23. Denison, M. S., Fisher, J. M., and Whitlock, J. P., Jr. (1988) J. Biol. Chem. 263, 17221–17224
24. Yee, F., and Denison, M. S. (1992) Biochemistry 31, 5060–5067
25. Israel, D. I., and Whitlock, J. P., Jr. (1984) J. Biol. Chem. 259, 5400–5402
26. Sable, A. D., and Bjeldanes, L. F. (1989) Carcinogenesis 10, 629–634
27. Lubet, R. A., Mayer, R. T., Cameron, J. W., Nims, R. W., Burke, M. D., Wolff, T., and Guengerich, F. P. (1985) Arch. Biochem. Biophys. 238, 43–48
28. Dixon, M. (1953) Biochem. J. 55, 170–171
29. Seed, B., and Sheen, J. Y. (1988) Gene (Amst.) 67, 271–277
30. Hankinson, O., Anderson, R. D., Birren, B., Sander, F., Negishi, M., and Nebert, D. W. (1985) J. Biol. Chem. 260, 1790–1795
31. Miller, A. G., Israel, D., and Whitlock, J. P., Jr. (1983) J. Biol. Chem. 258, 3523–3527
32. Daujat, M., Clair, P., Astier, C., Fabre, I., Pineau, T., Yerle, M., Gellin, J., and Maurel, P. (1991) Eur. J. Biochem. 200, 501–510
33. Kimura, S., Gonzalez, F. J., and Nebert, D. W. (1986) Mol. Cell. Biol. 6, 1471–1477
34. Silver, G., and Krauter, K. S. (1988) J. Biol. Chem. 263, 11802–11807
35. Caspar, D. S., Bayoum, K. W., Merchant, S. N., Chalberg, S. C., and Fagan, J. B. (1988) J. Biol. Chem. 263, 8671–8676
36. Whitlock, J. P., Jr., Denison, M. S., Fisher, J. M., and Shen, E. S. (1989) Mol. Biol. Med. 6, 169–178
37. Israel, D. I., and Whitlock, J. P., Jr. (1983) J. Biol. Chem. 258, 10390–10394
38. Takahashi, N., Dashwood, R. H., Bjeldanes, L. F., Bailey, G. S., and Williams, D. E. (1995) Food and Chemical Toxicology 33, 111–120
39. Riddick, D. S., Huang, Y., Harper, P. A., and Okey, A. B. (1994) J. Biol. Chem. 269, 12118–12128
40. Xu, L.-C., and Bresnick, E. (1990) Biochem. Pharmacol. 40, 1399–1403
41. Pesonen, M., and Andersson, T. (1991) Xenobiotica 21, 461–471
42. Christou, M., Stewart, P., Pottinger, L. H., Fahl, W. E., and Johnson, C. R. (1990) Carcinogenesis (Lond.) 11, 1691–1698
43. Astrov, B., and Safe, S. (1989) Toxicology 59, 285–296
44. Siess, M.-H., Bon, A. L., Suschetet, M., and Rat, P. (1990) Food Addit. Contam. 7, 5178–5181
45. Lesca, P., Raffdinariova, E., Lecointe, P., and Mansuy, D. (1979) Chem. Biol. Interactions 24, 189–198
46. Cai, Y., Bennett, D., Nair, R. V., Ceska, O., Ashwood-Smith, M. J., and DiGiovanni, J. (1992) Chem. Res. Toxicol. 6, 872–879
47. Murray, M., and Reidy, G. F. (1990) Pharmacol. Rev. 42, 85–101
48. Lesca, P., Lecointe, P., Paoletti, C., and Mansuy, D. (1978) Biochem. Pharmacol. 27, 1203–1209
49. Klem, M. H., Poulsen, L., and Gustafsson, J.-Å. (1994) J. Biol. Chem. 269, 5137–5144