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Ah RECEPTOR BINDING PROPERTIES OF INDOLE CARBINOLS AND INDUCTION OF HEPATIC ESTRADIOL HYDROXYLATION

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Abstract—The effect of route of administration on the ability of indole-3-carbinol (I3C), an anti-carcinogen present in cruciferous vegetables, to induce estradiol 2-hydroxylase (EH) in female rat liver microsomes was investigated and compared to that of its main gastric conversion product, 3,3′-diindolylmethane (DIM). This dimer was more potent than I3C after either oral or intraperitoneal administration and was also a better *in vitro* inhibitor of EH in control and I3C-induced hepatic microsomes. The induction of both CYP1A1 and 1A2 in about equal amounts by I3C and DIM as well as of CYP2B1/2 was demonstrated using monoclonal antibodies. DIM, isosafrole, β-naphthoflavone, 3-methylcholanthrene and naringenin added *in vitro* inhibited EH strongly in induced microsomes but gestodene was a better inhibitor of estrogen 2-hydroxylation in liver microsomes from untreated female rats. The binding affinities of I3C and DIM to the Ah receptor were compared to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by competition studies, and the IC_{50} values were shown to be 2.0×10^{-9} M, 5.0×10^{-5} M and 2.3×10^{-3} M for TCDD, DIM and I3C, respectively. The ability of I3C or DIM to cause *in vitro* transformation of the Ah receptor to a form able to bind to the dioxin-responsive element-3 (DRE3) was compared to that of TCDD and shown to parallel their abilities to compete for binding of [^{3}H]TCDD to the Ah receptor. These experiments confirm and extend the proposals that dietary indoles induce specific cytochrome P450s in rat liver by a mechanism possibly involving the Ah receptor. The induced monooxygenases, in turn, increase the synthesis of 2-hydroxylated estrogens in the competing pathways of 2- and 16α-hydroxylation which decreases the levels of 16α-hydroxyestrone able to form stable covalent adducts with proteins including the estrogen receptor. Such steroid–protein interaction has been correlated with mammary carcinogenesis.

It is now well established that indole-3-carbinol (I3C¶) and other compounds present in cruciferous vegetables (cabbage, broccoli, Brussels sprouts) possess anticarcinogenic properties and reduce the formation of covalent adducts of certain carcinogens with DNA [1–4]. Increased consumption of these vegetables is associated with reduced tumor incidence in humans [5, 6] and experimental animals [7–9]. I3C is a natural antioxidant in the human diet and, as such, may intervene in toxicological or carcinogenic processes that are mediated by electrophilic or free radical mechanisms [10], acting as a scavenger [11]. However, this action may be indirect because I3C is known to be converted irreversibly to its dimer 3,3′-diindolylmethane (DIM) and polymers under relatively mild conditions in the test tube [12] and also in the stomach [13, 14]. It has also been suggested [3, 15, 16] that induction of cytochrome P450 (P450) monooxygenase by indoles is associated with their preventive effects on neoplasia including hormone-dependent breast cancer. Thus, it has been shown recently that the oral administration of I3C to female mice [9] and rats [17] increases the ability of their liver microsomes to convert estradiol to its catechol derivative by estrogen 2-hydroxylase (EH). Alterations in the levels of specific P450 enzymes catalyzing the competing pathways of 2- and 16α-hydroxylation [18, 19] have been shown to be linked to mammary tumor formation [9, 20, 21]. 16α-Hydroxyestrone, unlike estrone or estradiol, can form stable adducts with proteins [22, 23] and the nuclear estrogen receptor [24, 25]. In addition, this estrogen metabolite induces genotoxic damage and aberrant proliferation in mouse mammary epithelial cells [26].

The objectives of this study were as follows: (a) to compare the dose-dependent induction of EH activity by I3C and DIM following oral or intraperitoneal administration, (b) to identify the P450 isozymes induced by I3C and DIM, (c) to compare I3C and DIM as *in vitro* inhibitors of EH activity, (d) to determine the relative contribution that induced P450 isozymes make to EH activity, (e) to compare the abilities of I3C and DIM to compete with [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-

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¶ Abbreviations: I3C, indole-3-carbinol; EH, estrogen 2-hydroxylase; DIM, 3,3′-diindolylmethane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; 3MC, 3-methylcholanthrene; β-NF, β-naphthoflavone; E_2, estradiol-17β; 2-OHE_2, 2-hydroxyestradiol-17β; DMSO, dimethyl sulfoxide; and DRE3, dioxin-responsive element-3.

dioxin ([^{3}H]TCDD) for binding to the cytosolic Ah receptor [27, 28], and (f) to compare the abilities of I3C and DIM to transform the Ah receptor to its DNA-binding form.

MATERIALS AND METHODS

Chemicals and reagents. [2-^{3}H]Estradiol (21.5 Ci/mmol) and [4-^{14}C]estradiol (57 mCi/mmol), purchased from New England Nuclear (Boston, MA), were shown by chromatography and autoradiography to be free of radioactive impurities. DIM was prepared in warm distilled water under N_2 by the method of Leete and Marion [12]. The product was recrystallized from benzene and found to be >95% pure by GC-MS. I3C, 3-methylcholanthrene (3MC), ascorbic acid and NADP were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]TCDD (31 Ci/mmol at the time of use) and unlabelled 2,3,7,8-tetrachlorodibenzofuran (TCDF) were gifts from Dr. S. Safe (Texas A & M University). Unlabelled TCDD was purchased from KOR Isotopes (Cambridge, MA). Adenosine 5′-[γ-^{32}P]triphosphate (>5000 Ci/mmol) was obtained from Amersham Canada Ltd. (Oakville, Ontario). T4 polynucleotide kinase was purchased from Pharmacia Canada Inc. (Baie d'Urfe, Quebec). Poly-deoxy-inosinic-deoxycytidylic acid (poly[d(I-C)]) was purchased from Boehringer Mannheim (Laval, Quebec). Monoclonal antibodies prepared against purified 3MC-induced rat liver P450 (Mab 1-7-1) and against phenobarbital-induced rat liver P450 (Mab 2-66-3) were a gift of Drs. H. V. Gelboin and S. S. Park (Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, MD). Gestodene (13-ethyl-17β-hydroxy-18,19-din-or-17α-pregna-4,15-dien-20-yn-3-one) was provided by Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN) and Dr. H. Kuhl (Johann Wolfgang Goethe University, Frankfurt-on-Maine).

Treatment of animals. Mature female (175–200 g) Sprague–Dawley rats (Charles River, St. Constant, Quebec, Canada) were maintained under standard conditions of light (6:00 a.m. to 8:00 p.m.) and temperature (21–22°) on a diet of Purina laboratory chow (Ralston–Purina, St. Louis, MO) and water *ad lib*. The animals were given I3C, DIM or 3MC in sesame oil by stomach tube and were killed 20 hr later by cervical dislocation after CO_2 anesthesia. Control animals received sesame oil alone.

Preparation of liver microsomal fraction and incubation. A 10% (w/v) homogenate of the liver was prepared in 0.25 M sucrose using a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 8000 *g* for 15 min and a microsomal fraction was obtained from the supernatant by centrifuging at 105,000 *g* for 1 hr, washing with 0.5 mL of 0.25 M sucrose, and resuspending the pellet in sucrose. The microsomes, derived from 200 mg of original tissue/mL, could be stored at −70° for several months without significant loss of activity. Protein was determined by the method of Lowry *et al.* [29].

The ^{3}H- or ^{14}C-labelled steroids (4.6 or 18.4 μM; 7–9 × 10^4 dpm) were incubated with constant shaking at 37° with the resuspended microsomes from 50 mg of liver (1.0 to 1.1 mg protein), and NADPH (0.3 mM) in 0.1 M potassium phosphate (pH 7.4), in a total volume of 4 mL. Inhibitors were added in ethanol (10 μL) which was also present in the control tubes. The solution was then extracted three times with equal volumes of diethyl ether, dried over anhydrous Na_2SO_4, and evaporated to dryness under N_2 at 25°.

Separation of metabolites and determinations of ^{3}H release into ^3H_2O. For the incubation with [4-^{14}C]estradiol, the residue was dissolved in ethanol (0.25 mL) and, after the addition of 5 μg of the catechol estrogen as carrier to a portion of the extract, the products were separated by thin-layer chromatography on silica gel using benzene: heptane:ethyl acetate (5:2:3, by vol.) or chloroform: ethyl acetate (3:1, v/v) and localized by autoradiography. The areas containing estradiol-17β (E_2) and 2-hydroxyestradiol-17β (2-OHE_2) were scraped directly into vials for determination of ^{14}C radioactivity by scintillation counting. The release of ^{3}H into ^3H_2O was determined by a radiometric assay described previously [30].

Preparation of liver cytosol. Male Sprague–Dawley rats weighing 200–250 g were obtained from Camm Research Lab Animals (Wayne, NJ). Hepatic cytosol was prepared in HEGD buffer [25 mM HEPES, 1.5 mM EDTA, 10% (v/v) glycerol, 1.0 mM dithiothreitol, pH 7.4] as described previously [31]. Protein concentration was determined by the method of Bradford [32]. The cytosol used in these experiments contained 157 fmol/mg protein specific [^{3}H]TCDD binding sites (B_{max}) and an apparent equilibrium dissociation constant (K_d) for [^{3}H]TCDD of 2.7 nM (as determined by Woolf analysis of receptor saturation data).

Ah receptor competitive binding studies. Cytosol (0.5 mL, 5 mg protein/mL) was incubated with 5 nM [^{3}H]TCDD in the absence or presence of unlabelled competitor for 1 hr at 4°. Dimethyl sulfoxide (DMSO) was used as the solvent for the radioligand and all competitors. Radioligand and competitors were added to the cytosolic incubation in 5-μL volumes. Various concentrations of unlabelled TCDD, I3C, and DIM were tested for their abilities to compete with [^{3}H]TCDD for specific Ah receptor binding. In all experiments, nonspecific binding was determined by incubating cytosol with 5 nM [^{3}H]-TCDD in the presence of a 100-fold molar excess of TCDF. After incubation, unbound and loosely bound radioligand was removed by treating samples with dextran-coated charcoal (1 mg/mg protein).

Sucrose density gradient analysis. Samples were analyzed by density gradient centrifugation using the vertical tube rotor technique [33]. Aliquots (300 μL) of charcoal-treated cytosol were layered onto linear (10–30%) sucrose gradients and then centrifuged at 4° for 2 hr at 372,000 g_{av}. After centrifugation, twenty-five fractions (200 μL each) were collected from each gradient (ISCO model 640 fractionator) and the radioactivity in each fraction was determined by liquid scintillation counting. Bovine serum albumin (4.4 *S*) and catalase (11.3 *S*) labelled with [^{14}C]formaldehyde were included in each gradient as internal sedimentation markers.

The IC_{50} (concentration of competitor required

to reduce specific binding of [^{3}H]TCDD by 50%) for each competitor was determined by interpolation from a plot of specific binding versus $\log_{10}$[competitor].

Gel retardation assay. A complementary pair of synthetic DNA fragments with sequences 5'-GATCTGGCTCTTCTCACGCAACTCCG-3' and 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3', containing the Ah receptor binding site of dioxin-responsive element-3 (DRE3) from the 5'-upstream region of the mouse *Cyp1a-1* gene, was synthesized (Dalton Biochemical, Toronto, Ontario), purified by high performance liquid chromatography, annealed, and radiolabelled with [γ-^{32}P]ATP using T4 polynucleotide kinase as described [34].

Cytosol (25 μL, 9 mg protein/mL) was incubated with DMSO or various concentrations of [^{3}H]TCDD, I3C, or DIM for 2 hr at 22°. DMSO was used as the solvent for all ligands, and the compounds were added to the cytosolic incubation in 1-μL volumes. The gel retardation assay, which measures the ability of a ligand to transform the Ah receptor to its specific DRE3-binding form, was performed essentially as described by Denison and Yao [35]. "Transformed" cytosol (80 μg protein) was incubated with poly [d(I-C)] (0.25 μg) for 15 min at room temperature. Following addition of the ^{32}P-labelled DRE3 sequence (78,000 cpm, 3 ng), the mixture was incubated for a further 15 min at room temperature. The binding reaction was carried out in HEGD buffer containing 80 mM NaCl. Protein–DNA complexes were analyzed by nondenaturing polyacrylamide gel electrophoresis and autoradiography. The ligand-inducible radiolabelled band was excised from the dried gel and ^{32}P was quantitated by liquid scintillation counting. The difference in radioactivity between ligand- and DMSO-treated samples was used to calculate the amount of ligand-inducible DRE3 binding.

The EC_{50} (concentration of ligand required to cause 50% of maximum transformation) for TCDD was determined by interpolation from a plot of DRE3 binding versus $\log_{10}$[ligand].

Immunoblotting. Liver microsomes (2–20 μg protein) were kept for 5 min at 95° in the loading buffer [0.25 M Tris–HCl (pH 6.8), 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate (SDS) and 0.001% (w/v) bromophenol blue] and electrophoresed on standard 9 × 14 cm 10% polyacrylamide gels [36] at 35 V for 18 hr. Catalase and prestained molecular weight markers (mol. wt 14,300–200,000, Amersham) were run at the same time. The gel was transblotted for 4 hr at 35 V in 22 mM Tris base:190 mM glycine:20% (v/v) methanol (pH 8.2) onto Hybond-ECL nitrocellulose, pore size: 0.45 μm (Amersham).

Non-specific binding to the blots was prevented by preincubation in 5% (w/v) skim milk powder–Tris saline [20 mM Tris–HCl (pH 7.6)/137 mM NaCl] containing 5% (v/v) Tween-20 (TBS-T) for 1 hr at 22° followed by washing (3×) with TBS-T. The blots were then incubated for 18 hr with the diluted primary antibody (Mab 1-7-1 or Mab 2-66-3) in TBS containing 2.5% (w/v) skim milk powder and washed again (3×) with TBS-T before addition of the secondary antibody (sheep antimouse Ig) linked to horseradish peroxidase (Amersham) in TBS containing 2.5% (w/v) skim milk powder. They were incubated for 1 hr and washed (3×) with TBS-T, and the protein–antibody complex was visualized using an enhanced chemiluminescence detection kit (Amersham) based on the oxidation of luminal by the peroxidase. Catalase was located by staining with Ponceau S.

RESULTS

Dose–response experiments. The dose–response data (Table 1) indicate that DIM, one of the putative gastric conversion products of I3C, was more effective than the parent compound in inducing EH in rat liver microsomes. Thus, DIM produced an

Table 1. Effect of treatment with indole-3-carbinol (I3C) or 3,3'-diindolylmethane (DIM) on the formation of ^3H_2O from [2-^{3}H]estradiol or [4-^{14}C]2-hydroxyestradiol from [4-^{14}C]estradiol by female rat liver microsomes

Treatment	Dose (mg)	^{3}H transfer into ^3H_2O (%)		Formation of [4-^{14}C]2-OHE$_2$ (%)	
		Oral	i.p.	Oral	i.p.
Control		33.0 ± 2.3	30.5 ± 3.0	9.1 ± 1.8	7.3 ± 1.4
I3C	20	64.2 ± 2.2	40.1 ± 2.4	21.5 ± 1.7	13.3 ± 1.1
	10	49.6 ± 2.4	39.2 ± 3.9	18.7 ± 2.2	14.6 ± 1.1
	5	43.9 ± 6.1	37.2 ± 3.4	16.0 ± 1.3	14.6 ± 1.4
DIM	5	60.4 ± 1.6	61.9 ± 2.4	19.5 ± 2.0	21.9 ± 2.0
	1	38.9 ± 3.9	49.6 ± 4.6	14.7 ± 0.4	16.3 ± 1.1
	0.3	28.5 ± 1.6	48.3 ± 4.4	8.2 ± 0.7	9.6 ± 1.6

Microsomes from 50 mg liver were incubated for 1 hr at 37° with [2-^{3}H]E$_2$ or [4-^{14}C]E$_2$ (18.4 μM) and NADPH (0.3 mM) in 4 mL potassium phosphate (0.1 M), pH 7.4. Ascorbate (2 mM) was added when [4-^{14}C]E$_2$ was used as substrate. The amount of ^3H_2O formed during the reaction and the yield of [4-^{14}C]2-OHE$_2$ separated by TLC was determined as described in Materials and Methods. Values are the means ± SEM from 6–9 rats (^3H_2O release) or 5–7 rats (formation of [4-^{14}C]2-OHE$_2$).

increase in the activity of this enzyme at lower doses and, unlike I3C, it was more effective by the i.p. than by the oral route of administration. This suggests a need for conversion of I3C to its dimer for enhanced inducing activity but does not exclude the formation of higher polymers with even greater inducing potency [37]. The formation of ^3H_2O from [2-^{3}H]E_2 was greater than the yield of 2-hydroxyestradiol from [4-^{14}C]E_2, but this can be accounted for by procedural losses in isolating the catechol estrogen and also the need to add ascorbic acid as a protective agent for the labile product. Ascorbate has been shown previously to have some inhibitory action on 2-hydroxylation as measured by the release of ^{3}H from [2-^{3}H]E_2 by rat liver microsomes and ^{3}H can also be lost by covalent binding to glutathione and protein at C-2 of the steroid after activation by hydroxylation at C-4 [38]. Treatment with I3C or DIM did not result in a significant increase in total liver protein.

Immunoblotting. The abilities of I3C and DIM to induce EH activity and CYP1A immunoreactivity were compared to those of 3MC, a classical Ah receptor ligand and CYP1A1 inducer, and isosafrole, a classical CYP1A2 inducer. Differential induction of CYP1A isozymes by I3C, DIM and 3MC was shown by Western blot analysis using a monoclonal antibody (Mab 1-7-1) that recognizes CYP1A1 and CYP1A2 (Fig. 1). I3C (20 mg, oral) and DIM (10 mg, i.p.) induced CYP1A1 and CYP1A2 in about equal amounts, whereas 3MC and β-naphthoflavone (β-NF) induced primarily CYP1A1 and isosafrole mainly CYP1A2, also present in small amounts in the untreated control animals. The molecular weights of CYP1A1 and 1A2 have been reported to be 57,000 and 56,000, respectively [39]. In addition, oral I3C induced slightly less CYP2B1 and/or CYP2B2, both of which are recognized by Mab 2-66-3 (data not shown). However, these studies were intended to identify the species of P450 that were induced and not to quantitate the level of induced P450 protein.

Effects of inhibitors. The abilities of I3C and DIM to inhibit EH activity *in vitro* were compared with those of compounds with known P450 isozyme selectivity. A good correlation has been shown previously [40] between the ability of a compound to act as a hydroxylase inducer *in vivo* and an inhibitor *in vitro*. Isosafrole is a selective CYP1A2 ligand, whereas βNF and 3MC would be expected to inhibit CYP1A1-mediated reactions. Naringenin (4′,5,7-trihydroxyflavanone) and gestadene have been reported to inhibit reactions catalyzed by CYP3A subfamily forms [41, 42]. All the compounds tested inhibited the 2-hydroxylation of E_2 by liver microsomes but the parent indole, I3C, was the least active (Table 2). Most of the compounds, which were inducers of P450, showed greater inhibitory effects in microsomes from I3C-treated than untreated female rats. Gestodene was the most potent inhibitor tested and was more active with control (63% inhibition at 1 μM) than with microsomes from I3C-treated animals (21% inhibition at 1 μM) upon incubation with [2-^{3}H]E_2 (4.6 μM) (not shown in Table 2).

Competition for the Ah receptor. The abilities of TCDD, I3C, and DIM to compete for specific binding of [^{3}H]TCDD to rat hepatic cytosolic Ah receptor were examined and representative sucrose gradient profiles are shown in Fig. 2. In all cases, the cytosolic Ah receptor sedimented in the 9 *S* region (fractions 10–16) of the gradients. Binding of [^{3}H]TCDD was eliminated in the presence of a 100-fold molar excess of unlabelled TCDF (Fig. 2), confirming the ligand specificity of the 9 *S* Ah receptor. DIM (Fig. 2) and I3C (data not shown) also reduced [^{3}H]TCDD binding to the Ah receptor. A summary of the ability of the three unlabelled compounds to compete for specific [^{3}H]TCDD binding to the Ah receptor is presented in Fig. 3. The following IC_{50} values were determined: TCDD, 2.0×10^{-9} M; DIM, 5.0×10^{-5} M; I3C, 2.3×10^{-3} M.

Transformation of the Ah receptor to a DRE3-binding form. A comparison of the abilities of TCDD, I3C, and DIM to cause concentration-dependent *in vitro* transformation of the Ah receptor to a DRE3-binding form is shown in Fig. 4. TCDD was found to be highly potent (EC_{50} = 3 nM) and efficacious (maximum transformation = 25 fmol/mg protein, or 16% of total Ah receptor sites determined previously by [^{3}H]TCDD binding) in promoting transformation of the Ah receptor. In contrast, both I3C and DIM displayed greatly reduced potency and efficacy compared to TCDD. The abilities of these compounds to transform Ah receptor were not sufficient to allow the calculation of a reliable EC_{50} value, although their relative transformation potencies appeared to parallel their abilities to compete for binding of [^{3}H]TCDD to the Ah receptor.

DISCUSSION

These experiments support the hypothesis that I3C and other dietary indoles influence the level of monooxygenase activity by forming condensation products upon introduction into the acidic environment of the stomach [13]. Thus, DIM, the dimer of I3C, was more effective than the parent compound in increasing the hydroxylation of estradiol to its catechol derivative by rat liver microsomes. DIM evoked an inductive response when given at a dose of 5 mg/kg body weight i.p., whereas I3C administered by this route which bypasses the stomach was only active at higher doses. However, other acid-induced condensation products of I3C, such as indolo[3,2-*b*]carbazole, may be even more active [37].

We had observed previously (unpublished data) that the pattern of metabolites produced by liver microsomes, using 4-androstenedione as substrate, was also influenced by pretreatment with I3C or DIM. The pattern resembled more closely that produced by isosafrole, a classical CYP1A2 inducer, than that produced by the classical CYP1A1 inducers 3MC and βNF. It has also been shown recently that the mRNAs and proteins for both CYP1A1 and 1A2 as well as others (e.g. CYP2B1, 2B2, 2E1) were induced in rat liver after oral treatment with I3C or by using a diet which included freeze-dried broccoli [43, 44]. Our experiments using a monoclonal antibody (Mab 1-7-1) which recognizes both CYP1A1

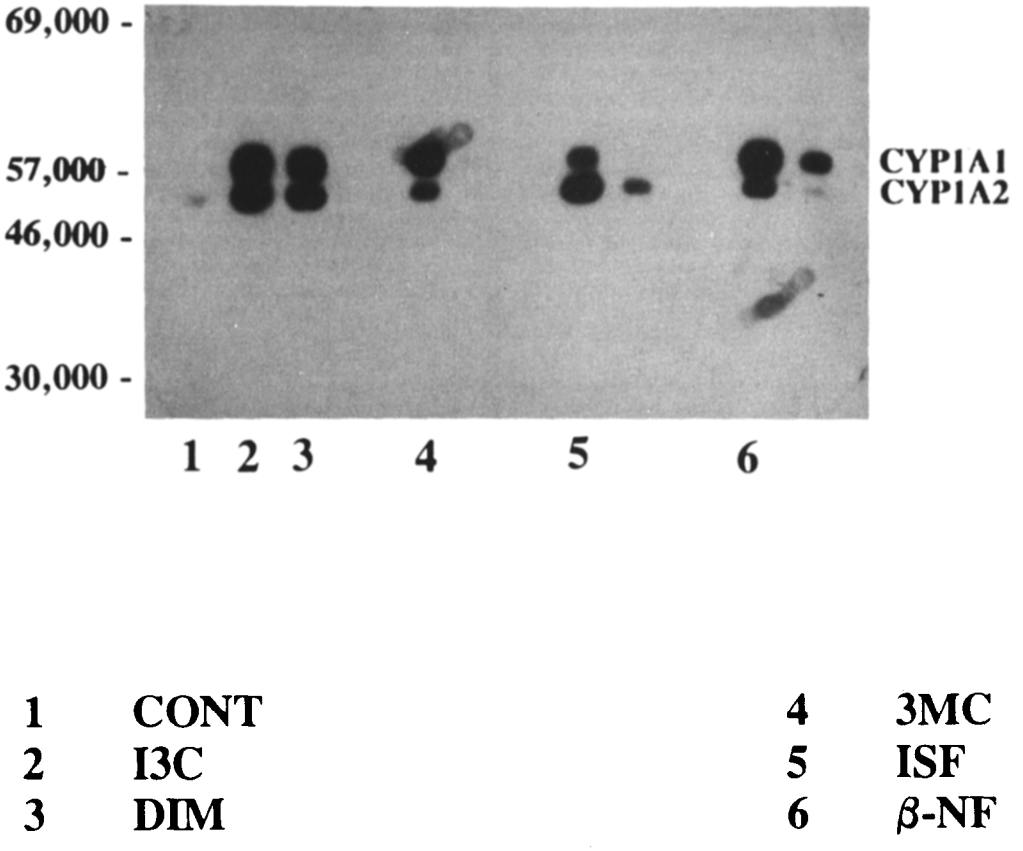

Fig. 1. Western blots of cytochrome P450 isozymes. Microsomal proteins from control and treated rats were separated by SDS–polyacrylamide gel electrophoresis and then subjected to Western blotting with Mab-1-7-1 as described in Materials and Methods. Hepatic microsomal fractions are as follows: lane 1, control (20 μg protein); lane 2, I3C (20 mg, oral; 20 μg protein); lane 3, DIM (10 mg, i.p., 20 μg protein); lane 4, 3-methylcholanthrene (10 mg, oral; 5 μg protein); lane 5, isosafrole (ISF) (30 mg, i.p., daily × 3; 5 and 1 μg protein); lane 6, β-NF (10 mg, i.p., daily × 3; 5 and 1 μg protein).

Table 2. Effect of inhibitors on the formation of ^3H_2O from [2-^{3}H]estradiol by liver microsomes of female rats treated orally with I3C

	Control		I3C-treated	
Additions	^3H_2O (%)	Inhibition (%)	^3H_2O (%)	Inhibition (%)
Control	16.9 ± 1.5		48.9 ± 3.3	
I3C	14.6 ± 0.2	13	33.9 ± 0.6	30
DIM	7.2 ± 0.4	57	13.5 ± 1.3	72
Isosafrole	12.0 ± 0.7	29	16.8 ± 1.5	65
β-Naphthoflavone	9.8 ± 2.4	42	11.7 ± 3.5	76
3-MC	12.2 ± 1.3	27	15.1 ± 1.0	69
Naringenin	8.8 ± 1.0	48	15.0 ± 0.1	69
Gestodene	4.3 ± 0.2	74	19.8 ± 1.1	59

Microsomes were incubated with [2-^{3}H]E_2 (4.6 μM) and the inhibitors (20 μM) for 15 min under the conditions described in Table 1. Treated rats received 20 mg of I3C orally. Values are the means ± SEM from 3 rats.

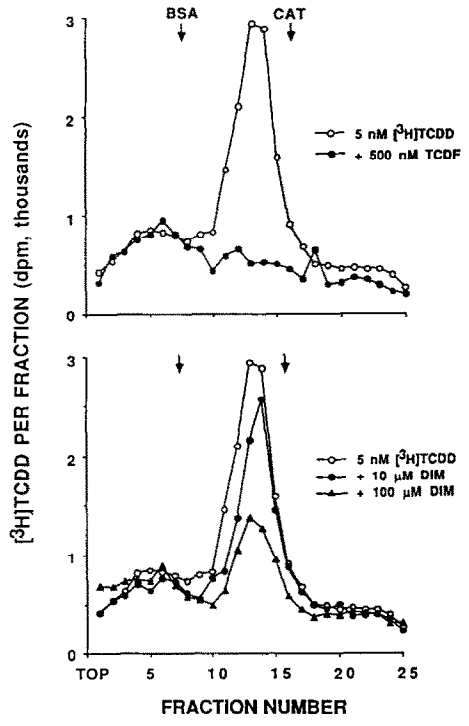

Fig. 2. Sucrose density gradient profiles demonstrating competition by various ligands for specific [^{3}H]TCDD binding sites in rat hepatic cytosol. Cytosol from rat liver prepared in HEGD buffer was incubated for 1 hr at 4° with 5 nM [^{3}H]TCDD in the absence or presence of a 100-fold molar excess of TCDF (upper) or a 2,000- or 20,000-fold molar excess of DIM (lower). Samples were analyzed by velocity sedimentation on sucrose gradients. The concentration of specific [^{3}H]TCDD binding sites in the 9 *S* region, detected with a subsaturating concentration of radioligand, was 98 fmol/mg protein in the absence of competitor. Arrows indicate the sedimentation positions of [^{14}C]formaldehyde-labelled BSA (4.4 *S*) and catalase (11.3 *S*).

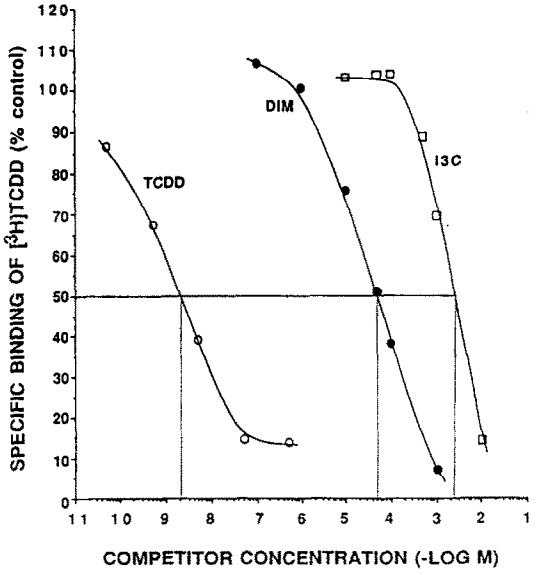

Fig. 3. Competition for specific binding of 5 nM [^{3}H]TCDD to rat hepatic Ah receptor by unlabelled TCDD, I3C, and DIM. Rat liver cytosol was incubated for 1 hr at 4° with 5 nM [^{3}H]TCDD in the absence or presence of various concentrations of unlabelled competitors. Samples were analyzed by velocity sedimentation on sucrose gradients. The mean concentration of specific [^{3}H]TCDD binding sites in the 9 *S* region, detected with a subsaturating concentration of radioligand, was 108 fmol/mg protein in the absence of competitor (=100%). Dotted lines indicate the interpolated IC_{50} value for each competitor.

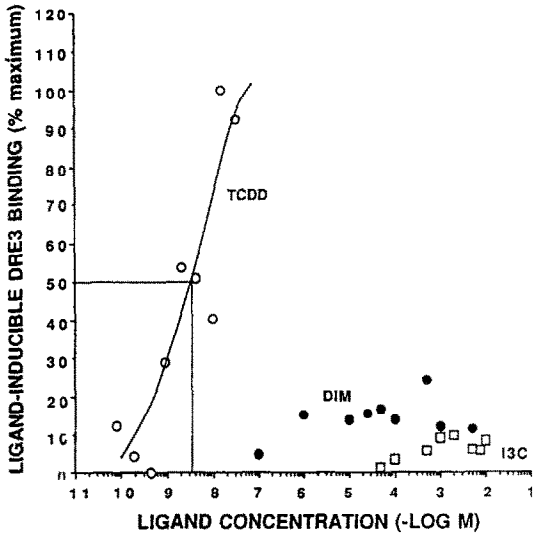

Fig. 4. Log concentration–response for *in vitro* transformation of the rat liver cytosolic Ah receptor to the DRE3-binding form by [^{3}H]TCDD, I3C, and DIM. Rat liver cytosol was incubated for 2 hr at 22° with DMSO or various concentrations of [^{3}H]TCDD, I3C, or DIM. The binding of transformed Ah receptor to [^{32}P]DRE3 was analyzed by gel retardation. The difference in radioactivity between ligand- and DMSO-treated samples was used to calculate the amount of ligand-inducible DRE3 binding. The maximum amount of ligand-inducible DRE3 binding was 25 fmol/mg protein (=100%).

and 1A2 [39] confirm these results and showed about equal induction of these isozymes. We have also demonstrated the induction of CYP2B1/2. This does not exclude the hepatic induction by indole carbinols of other P450s, known to catalyze the 2/4-hydroxylation of estradiol [19, 45].

Although the apparent binding affinities of I3C and DIM for the Ah receptor are low when compared to TCDD, the doses given may be sufficient to act through the Ah receptor [46–48] to increase gene expression. However, this will depend on the kinetics of uptake and distribution of the indoles to various organs when given *in vivo* and the local concentration that they might achieve near the receptor sites. Recently, Bjeldanes *et al.* [37] obtained very similar results in competition studies with I3C and DIM on the binding of [2-^{125}I]7,8-dibromodibenzo-*p*-dioxin to the Ah receptor prepared from C57BL/6J mouse liver cytosol. In addition, they found that indolo[3,2-*b*]carbazole, produced from I3C in very low amounts after oral intubation, had a binding affinity

approaching that of TCDD. They suggested that this compound and related condensation products were responsible for the enzyme-inducing effects of dietary I3C.

It is possible that the indoles act by stabilizing P450 mRNA or P450 protein or by a combination of these mechanisms. A post-transcriptional component has been proposed to account for the increase in CYP1A2 mRNA following treatment with inducers [49–51]. It has also been shown [43] that a mRNA corresponding to CYP1A2 is present and inducible by I3C in liver but not in colon, which is supported by the observation that accumulation of CYP1A2 mRNA after a broccoli diet does not occur in extrahepatic tissues [44]. In contrast, CYP1A1 mRNA is increased by I3C in both liver and colon [43].

In conclusion, these experiments confirm and extend the proposals [37, 47, 48] that dietary indoles induce specific P450s, in particular CYP1A1 and CYP1A2, in the liver by a mechanism involving the Ah receptor. These cytochromes, in turn, are able to increase the 2-hydroxylation of estrogens at the expense of the formation of 16α-hydroxyestrone [17, 52] and thus protect target cells from the deleterious actions of this steroid. 16α-Hydroxyestrone has been shown to function as an initiator and promoter of mammary cell transformation [26] and also to decrease greatly estrogen and progesterone receptor levels in MCF-7 cells [25]. In addition, it can increase the expression of oncogenes in this cell line.*

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