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Ligand-Independent Activation of Estrogen Receptor Function by 3,3′-Diindolylmethane in Human Breast Cancer Cells

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ABSTRACT. 3,3′-Diindolylmethane (DIM), a major *in vivo* product of acid-catalyzed oligomerization of indole-3-carbinol (I3C), is a promising anticancer agent present in vegetables of the *Brassica* genus. We investigated the effects of DIM on estrogen-regulated events in human breast cancer cells and found that DIM was a promoter-specific activator of estrogen receptor (ER) function in the absence of 17β-estradiol (E_2). DIM weakly inhibited the E_2-induced proliferation of ER-containing MCF-7 cells and induced proliferation of these cells in the absence of steroid, by approximately 60% of the E_2 response. DIM had little effect on proliferation of ER-deficient MDA-MB-231 cells, suggesting that it is not generally toxic at these concentrations. Although DIM did not bind to the ER in this concentration range, as shown by a competitive ER binding assay, it activated the ER to a DNA-binding species. DIM increased the level of transcripts for the endogenous pS2 gene and activated the estrogen-responsive pERE-vit-CAT and pS2-tk-CAT reporter plasmids in transiently transfected MCF-7 cells. In contrast, DIM failed to activate transcription of the simple E_2- and diethylstilbesterol-responsive reporter construct pATC2. The estrogen antagonist ICI 182780 (7α-[9-[(4,4,5,5,5-pentafluoropentyl)sulfonyl-]nonyl]-estra-1,3,5(10)-triene-3,17β-diol) was effective against DIM-induced transcriptional activity of the pERE-vit-CAT reporter, which further supports the hypothesis that DIM is acting through the ER. We demonstrated that ligand-independent activation of the ER in MCF-7 cells could be produced following treatment with the D1 dopamine receptor agonist SKF-82958 [(±)6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepinehydrobromide]. We also demonstrated that the agonist effects of SKF-82958 and DIM, but not of E_2, could be blocked by co-treatment with the protein kinase A (PKA) inhibitor H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide). These results have uncovered a promoter-specific, ligand-independent activation of ER signaling for DIM that may require activation by PKA, and suggest that this major I3C product may be a selective activator of ER function. BIOCHEM PHARMACOL **60**;2: 167–177, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. estrogen receptor; agonist; breast cancer; diindolylmethane; ligand independent

I3C§ (Fig. 1), a hydrolysis product of glucobrassicin found in *Brassica* plants, including turnips, kale, broccoli, Brussels sprouts, and cauliflower, is a potential cancer protective agent. Oral administration of I3C reduced BP-induced neoplasia of the forestomach and total covalent binding of BP and *N*-nitrosodimethylamine to hepatic DNA in mice [1–3]. In trout, oral I3C reduced aflatoxin B1-induced hepatocarcinogenesis when administered prior to and during carcinogen treatment [4]. Similarly, in rodents, oral I3C reduced DMBA-induced mammary tumor incidence by 70–80% [1, 5], and reduced by 50% the incidence and multiplicity of spontaneous mammary tumors [6]. In a screen of 90 potential chemopreventive agents in a series of six short-term bioassays relevant to initiation and post-initiation phases of carcinogenesis, I3C was found to be one of only eight compounds that tested positive in all assays [7]. Some evidence suggests, however, that whereas I3C mitigates mammary carcinogenesis, it may also enhance tumorigenesis in other organs under certain conditions. Thus, oral administration of high doses of I3C to rodents or trout following exposure to certain organ-selective carcinogens resulted in increased tumor incidence of the liver [8],

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§ *Abbreviations*: AhR, aryl hydrocarbon receptor; BP, benzo[*a*]pyrene; CAT, chloramphenicol acetyltransferase; CDK, cyclin-dependent kinase; CYP, cytochrome P450; DES, diethylstilbesterol; DIM, 3,3′-diindolylmethane; DMBA, dimethylbenzanthracene; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; EROD, ethoxyresorufin *O*-deethylase; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; I3C, indole-3-carbinol; ICZ, indolo[3,2-*b*]carbazole; and PKA, protein kinase A.

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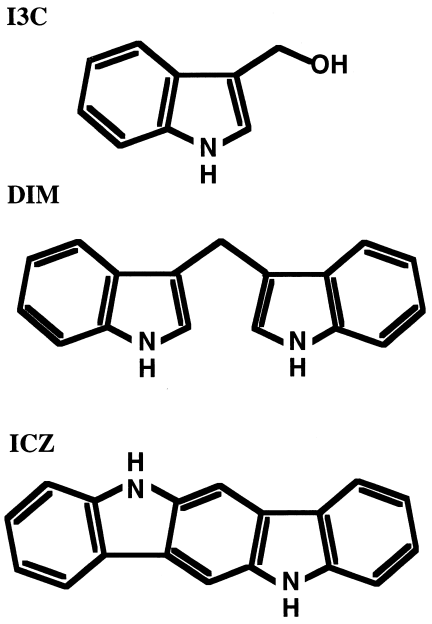

FIG. 1. Molecular structures of I3C, DIM, and ICZ.

colon [9], or thyroid gland [10]. These findings emphasize the importance of determining the mechanism of action of I3C before it is used in long-term cancer prophylaxis.

In a detailed analysis of the effects of I3C on cell proliferation and on components of the cell cycle regulation pathway, we showed that at high concentration, I3C produced a nearly complete but reversible G_1 cell cycle arrest in a manner that did not affect cell viability [11]. This arrest was accompanied by a selective repression of CDK6 expression, an inhibition of phosphorylation of retinoblastoma protein, and an increased expression of the CDK inhibitor proteins p21 and p27. Importantly, these effects were independent of the ER, since they also could be produced in an ER-deficient breast cancer cell line. These results suggest that I3C itself is not the cause of the antiestrogenic and tumor-promoting effects of orally administered I3C.

Of central importance to the *in vivo* biological activity of I3C is its facile conversion to other products on contact with acid. We have shown that the I3C induction of cytochrome P450 activity, and possibly other responses as well, is dependent on the route of administration. Whereas I3C fails to induce cytochrome P450 when administered by intraperitoneal injection into rats, thus bypassing contact with gastric acids, it is an active enzyme inducer when administered by oral intubation [12]. Acid treatment of I3C produces a mixture that is active by either route of administration. Analyses of this reaction mixture have indicated the presence of many minor oligomeric I3C products including ICZ, and several major products including DIM [13]; these products are also produced in the gastrointestinal tracts of rodents following oral treatment with I3C [14, 15].

ICZ is a planar pentacyclic compound (Fig. 1) that, in contrast to I3C, binds with high affinity to the AhR and is both a potent (but transient) inducer of AhR-regulated CYP1A1 expression and an inhibitor of CYP1A1-dependent enzyme activity [16]. Since the persistent activation of AhR-dependent pathways is thought to be responsible for the tumor-promoting effects of ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [17], conversion of I3C to ICZ and other potent AhR ligands may contribute to the adverse chronic effects resulting from high doses of I3C. DIM inhibits carcinogen-induced mammary tumor formation in rats [1] and weakly induces hepatic CYP1A enzymes *in vivo* [5, 18]. DIM is a weak ligand for the AhR, an antagonist of AhR-mediated gene transcription [19], and an inhibitor of CYP1A1 enzyme activity [20], suggesting that activation of AhR-dependent pathways may not be important in the protective effects of DIM against mammary cancer.

In the present study, we investigated, in human breast cancer cell lines, the interactions of DIM with E_2-regulated biological events including cell proliferation, binding affinity to the ER, and transcriptional expression of an endogenous gene and of transiently transfected reporter genes. We demonstrated that DIM acts as an agonist of E_2 function. DIM antagonized weakly the effects of E_2 on cell proliferation and transcriptional activation of a reporter gene, and DIM activated these responses in the absence of E_2. Although DIM did not bind to the ER, it produced a clear concentration-related activation of the ER to a DNA-binding species. We showed that the effects of DIM on ER-dependent transcription are promoter specific and may result from a PKA-related mechanism of ER activation.

MATERIALS AND METHODS

Materials

DMEM, Opti-MEM, and Lipofectamine were supplied by Gibco/BRL. Phenol red-free DMEM, FBS, calf serum, tamoxifen, and E_2 were supplied by the Sigma Chemical Co. ICI 182780 (7α- [9-[(4,4,5,5,5-pentafluoropentyl]sulfonyl]nonyl]-estra-1,3,5 [10]-triene-3,17β-diol) was supplied by Tocris. [γ-^{32}P]ATP and [^{3}H]acetyl-CoA were supplied by New England Nuclear. SKF-82958 [(±)6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepinehydrobromide] was purchased from RBI, and H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) was obtained from LC Laboratories. DIM was prepared from I3C as described [12–14] and recrystallized in toluene. All other reagents were of the highest grade available.

Cell Culture

The human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231, obtained from the American Type Culture Collection, were grown as adherent monolayers in DMEM, supplemented to 4.0 g/L of glucose and 3.7 g/L of sodium bicarbonate, in a humidified incubator at 37° and 5% CO_2,

and passaged at approximately 80% confluence. Cultures used in subsequent experiments were at less than 25 passages.

Cell Proliferation

Before the beginning of the treatments, cells were depleted of estrogen for 7–10 days in medium composed of DMEM base without phenol red (Sigma), with 4 g/L of glucose, 3.7 g/L of sodium bicarbonate, and 5% calf serum twice stripped in dextran-coated charcoal and microfiltered, supplemented with non-essential amino acids (Gibco), 2 mM glutamine, and 10 ng/mL of insulin. During the depletion period, medium was changed every other day. Treatments were administered by the addition of 1 μL of 1000x solution in DMSO/mL of medium. Once the treatment period started, medium was changed daily to counter possible loss of readily metabolized compounds.

Cell Counting

Cells were harvested by trypsinization and resuspended in complete medium. Aliquots were diluted 50-fold in Isoton II (Coulter Corp.), and 500-μL duplicates were counted in a model Z1 Coulter particle counter and averaged.

ER Binding Assay

Rat uterine cytosol was prepared as described previously [21]. Briefly, 2.5 g of uterine tissue from five Sprague–Dawley rats (12 weeks old) was excised and placed on ice. The fresh tissue was homogenized with 30 mL of ice-cold TEDG buffer (10 mM Tris, pH 7.4, 1.5 mM EDTA, 1 mM DTT, 10% glycerol) using a Polytron apparatus at medium speed for 1 min on ice. The homogenate was centrifuged at 1000 *g* for 10 min at 4°. The supernatant solution was transferred to ultracentrifuge tubes and centrifuged at 100,000 *g* for 90 min at 4°. The supernatant solution was divided into 1.0-mL aliquots, quickly frozen in a dry-ice/ethanol bath, and stored at −80°. Protein concentration of the uterine cytosol was 3 mg/mL, as measured by the Bradford assay using BSA as the standard. For each competitive binding assay, 5 μL of 20 nM [^{3}H]E_2 in 50% ethanol, 10 mM Tris, pH 7.5, 10% glycerol, 1 mg/mL of BSA, and 1 mM DTT was placed in a 1.5-mL microcentrifuge tube. Competitive ligands were added as 1.0 μL of 100x solution in DMSO. After mixing, 95 μL of uterine cytosol was added, and the solutions were vortexed and incubated at room temperature for 2–3 hr. Proteins were precipitated by the addition of 100 μL of 50% hydroxylapatite slurry equilibrated in TE (50 mM Tris, pH 7.4, 1 mM EDTA) and incubated on ice for 15 min with vortexing every 5 min to resuspend hydroxylapatite. The pellet was washed with 1.0 mL of ice-cold wash buffer (40 mM Tris, pH 7.4, 100 mM KCl), and centrifuged for 5 min at 10,000 *g* at 4°. The supernatant was aspirated carefully, and the pellet was washed two more times with 1.0 mL of wash buffer. The final pellet was resuspended in 200 μL ethanol and transferred to a scintillation vial. The tube was washed with another 200 μL ethanol, which then was added to the same counting vial. A negative control contained no uterine cytosol. Nonspecific binding was determined using 100-fold (0.1 μM) excess unlabeled E_2. Relative binding affinities were calculated using the concentration of competitor needed to reduce [^{3}H]E_2 binding by 50% as compared with the concentration of unlabeled E_2 needed to achieve the same result.

CYP1A1 Activity

CYP1A1 activity was measured by the EROD assay as described previously [16]. Briefly, after the 18- to 24-hr treatment of near-confluent (80%) cultures in 60-mm diameter dishes, cells were trypsinized and resuspended in 5 mL PBS. The cells and the substrate solution were preincubated at 37°. An aliquot of the cell suspension was counted to obtain the cell number, and 1.5 mL of the cell suspension was placed into a fluorometer cuvette, followed by the addition of 0.5 mL of 2.5 μM ethoxyresorufin (Sigma). The reaction mixture was mixed by inversion of the cuvette, and the fluorescence produced was recorded at the excitation wavelength of 510 nm and the emission wavelength of 586 nm with a 20-nm slit width using a Perkin-Elmer 650–10S spectrofluorometer at a calibrated chart speed. A standard curve was obtained using different amounts (0, 2.5, 5, 7.5, and 10 pmol) of resorufin (Sigma) added to the control cells in a total volume of 2.0 mL. The enzyme activity was presented as picomoles resorufin produced per minute per million cells.

Plasmid Reporters and Expression Vectors

The ER-responsive CAT reporter plasmids pERE-vit-CAT and pATC2 [22] were gifts from D. J. Shapiro (University of Illinois). pERE-vit-CAT contains the 5′-flanking and promoter region (−596 to 21) of the *Xenopus* vitellogenin-B1 gene, including two imperfect endogenous EREs (at −302 and −334) and an exogenous consensus ERE (GGT CACAGTGACC) inserted at position −359. The simpler reporter pATC2 contains two copies of the consensus ERE coupled 38 bp from the TATA box of the vitellogenin-B1 promoter (−42 to 14). The reporter plasmid pS2-tk-CAT, containing the promoter and flanking region (−1100 to 10) of the human pS2 gene [23], was obtained from P. Chambon (University of Strasbourg). The plasmid pCMV-hER, constitutively expressing a fully functional human ER [24], was a gift from B. S. Katzenellenbogen (University of Illinois). The transfection efficiency control vector, pCMVβ, constitutively expressing β-galactosidase, was obtained from the American Type Culture Collection.

Transient Transfections with Reporters

Transfections were performed by the lipofection method using Lipofectamine (Gibco). Cells were grown in 10% FBS–DMEM to sub-confluence and transferred to 6.0-cm Petri plates 24 hr before transfection. The plates were seeded with the appropriate number of cells to become 50–60% confluent at the time of transfection. For each 6-mm plate, 8 μL of Lipofectamine was diluted with 92 μL of Opti-MEM serum-free medium (Gibco). Plasmid DNA (0.1 to 1.0 μg) was diluted in 100 μL of serum-free medium. Lipid and plasmid dilutions were combined, mixed gently, and incubated at room temperature for 30–45 min. Meanwhile, the cells were washed with 4 mL of serum-free medium, and 2 mL of serum-free medium was added to each plate. Next, 200 μL of the lipid–DNA suspension was added to each plate and mixed gently. The plates were returned to the incubator for 5–6 hr, and 2 mL of medium containing 10% calf serum was added. The next day, the cells were refed with fresh depleted medium without phenol red (5% dextran-coated charcoal-stripped FBS), and the 48-hr treatments were started by the addition of 1 μL of 1000x solutions in DMSO/mL of medium. The transfection efficiency was determined using the constitutive galactosidase expression plasmid CMVβ in an identical set of plates and was found to be unaffected by the treatments.

CAT Assay

The CAT assay was performed using a modification of the phase extraction assay described by Seed and Sheen [25]. At the end of the 48-hr treatment period, the transfected cells were harvested by scraping with a rubber policeman, transferred with the medium to a conical 15-mL tube, centrifuged at 600 *g* for 2 min, resuspended in 1 mL of cold PBS, transferred to Eppendorf tubes, centrifuged at 600 *g* for 2 min, and washed in PBS a second time. Cell pellets were resuspended in 200 μL of 0.1 M Tris, pH 8.0, and lysed by three cycles of freeze/thaw treatment (alternating 5 min in a dry-ice/alcohol bath and 5 min in a 37° bath). Cell lysates were incubated at 65° for 15 min to inactivate acylases and centrifuged at 14,000 *g* for 8 min. A 165-μL aliquot of the cytosol was transferred to a 7-mL scintillation vial, and a 20-μL aliquot was reserved for determination of protein concentration by the Bradford assay. The substrate mixture (85 μL) was added to the scintillation vial to obtain final concentrations of 100 mM Tris–HCl, pH 8.0, 250 nmol chloramphenicol, 1 μCi [^{3}H]acetyl-CoA (200 mCi/mmol) in a total volume of 250 μL and mixed thoroughly. The organic scintillation fluid (4 mL) was added slowly to avoid mixing with the aqueous phase, and the vials were incubated at 37° for 1–2 hr or until sufficient counts were obtained.

RNA Extraction and Northern Blot Analysis

Cells were lysed by the addition of Tri-reagent (Molecular Research Center, Inc.), and chloroform was used for phase separation. After centrifugation, the aqueous upper phase was collected, and total RNA was precipitated by isopropanol, washed with 75% ethanol, and dissolved in diethylpyrocarbonate-treated water. Total RNA was electrophoresed on a 1.2% agarose gel containing 3% formaldehyde, using MOPS as the running buffer. The gel was washed gently with 10x SSC (sodium chloride/sodium citrate (buffer); 20x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) and blotted with a Zeta nylon membrane (Bio-Rad) overnight to transfer the RNA onto the membrane. The RNA was fixed to the membrane by UV cross-linking. The hybridization probes were labeled with ^{32}P using random primers and the pS2-cDNA and GADPH-cDNA plasmids provided by the American Type Culture Collection as the template. Hybridization and quantitation of results were performed as described previously [16]. Specific pS2 mRNA levels were normalized using GADPH as a standard.

Nuclear Extracts

Three near-confluent (80–90%) cultures of MCF-7 cells in 100-mm Petri dishes were used for each treatment. DIM or E_2 was added as 1 μL of 1000x solution in DMSO/mL of medium. After 2 hr of incubation at 37°, the plates were placed on ice, washed twice with 5 mL of hypotonic buffer (10 mM HEPES, pH 7.5), and incubated with 2 mL of HEPES for 15 min. Cells were harvested in 1 mL of MDH buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES, pH 7.5) with a rubber scraper, homogenized with a loose-fitting Teflon pestle, and centrifuged at 1000 *g* for 4 min at 4°. The pellets were washed twice with 3 mL of MDHK buffer (3 mM $MgCl_2$, 1 mM DTT, 0.1 M KCl, 25 mM HEPES, pH 7.5), resuspended in 1 mL of MDHK, and centrifuged at 600 *g* for 4 min at 4° in a microcentrifuge. The pellets were resuspended in 100 μL of HDK buffer (25 mM HEPES, pH 7.5, 1 mM DTT, 0.4 M KCl), incubated for 20 min on ice with mixing every 5 min, and centrifuged at 14,000 *g* for 4 min at 4°. Glycerol was added to the supernatants to a concentration of 10%, and aliquots of the nuclear extracts were stored at −80°.

Gel Mobility Shift Assay

The following two complementary 31-mer oligonucleotides:

5′-GATCCCAGGTCACAGTGACCTGAGCTAAAAT-3′ and 5′-GATCATTTTAGCTCAGGTCACTGTGACCTGG-3′ containing the palindromic ERE consensus motif (underlined) were annealed and 5′ end-labeled with [γ-^{32}P]ATP using T4 nucleotide kinase. The resulting labeled double-stranded DNA probe was purified on a Sephadex G50 spin-column, precipitated in ethanol, dissolved in TE buffer, and diluted in 25 mM HEPES, 1 mM DTT, 10% glycerol, 1 mM EDTA to contain approximately 25,000 cpm of ^{32}P/μL. Nuclear extracts (7 μg of proteins), were mixed with 90 ng poly(dIdC), 25 mM HEPES, 1 mM DTT, 10% glycerol, 1 mM EDTA, 160 mM KCl in a total

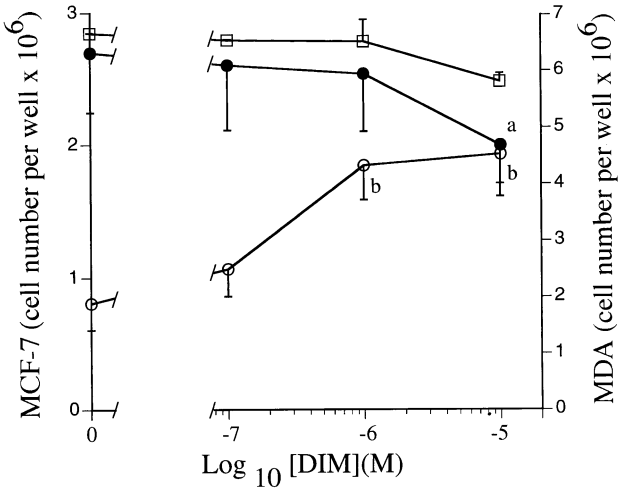

FIG. 2. Effect of DIM on proliferation of breast cancer cells. Estrogen-depleted MCF-7 cells were plated at a density of 10^5 cells/well in 6-well plates and treated with DIM at the concentrations indicated, in the presence (●) or absence (○) of E_2 (1 nM). MDA cells (□) grown in 10% FBS–DMEM were plated at a density of 10^5 cells/well in 6-well plates and treated with DIM at the concentrations indicated. Duplicate aliquots of cells from individual wells were counted after 7 days. Data from three identical wells are averaged. Results are shown as the means ± SD for four separate experiments. Key: (a) significantly reduced ($P < 0.05$) from the E_2-induced control; and (b) significantly different ($P < 0.05$) from the DMSO control.

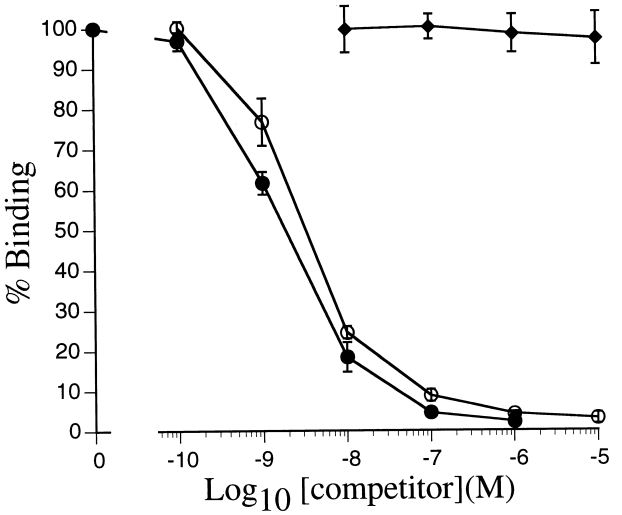

FIG. 3. Competitive binding to the ER. The binding of $[^3H]E_2$ (1 nM) to the ER from rat uterine cytosol was measured in the presence of the unlabeled competitors E_2 (●), ICI (○), and DIM (♦) at the concentrations indicated and reported as the percentage of binding in the absence of competitors. Results are shown as the means ± SD for four separate determinations. Relative binding affinities were calculated using the concentration of competitor needed to reduce $[^3H]E_2$ binding by 50% as compared with the concentration of unlabeled E_2 needed to achieve the same result.

volume of 21 μL. For antibody supershift experiments, 0.5 μg of monoclonal mouse IgG anti-human-ER (Santa Cruz Biotechnology) was added to the incubation mixture. After incubation for 15–20 min at room temperature, 4 μL (100,000 cpm) of end-labeled [^{32}P]ERE probe was added and incubated for another 15 min at room temperature. After the addition of 2.8 μL of 10x Ficoll loading buffer (0.25% bromophenol blue, 25% Ficoll type 400), 22-μL aliquots were loaded onto a pre-run, non-denaturing 4.0% polyacrylamide gel in TAE (67 mM Tris, 33 mM sodium acetate, 10 mM EDTA, pH 8.0) at 120 V for 2 hr. Finally, the gel was dried and autoradiographed.

RESULTS

DIM-Induced Growth of MCF-7 Cells in E_2-Depleted Medium

The effects of DIM on cell proliferation were examined in estrogen-treated and untreated MCF-7 cells over a 7-day time course. Treatment of cells with DIM in E_2-stripped medium produced a concentration-dependent increase in cell proliferation that at 10 μM reached a maximum of 60% of the growth produced in the absence of DIM in cells grown in complete, E_2-rich medium (Fig. 2). Co-treatment of cells with DIM (10 μM) in the E_2-rich medium, however, weakly inhibited estrogen-stimulated cell growth. DIM had no significant effect on proliferation of the ER-deficient breast cancer cell line MDA-MB-231 (Fig. 2). Taken together, these results suggested that DIM may affect breast tumor cell proliferation via an interaction with the ER and that DIM is not generally cytotoxic at the antiproliferative concentrations.

ER Affinity for DIM and Activation of ER Binding to the ERE

Based on our observation of a proliferative response, we assessed directly the ability of DIM to bind ER and activate its DNA-binding function. The relative binding affinity of DIM for the ER was measured by a competitive binding assay using 1.0 nM $[^3H]E_2$ as the labeled ligand (Fig. 3). The IC_{50} values, the concentrations of competitors necessary to displace the labeled ligand by 50%, were 2.0 and 4.0 nM for E_2 and ICI 182780 (ICI), respectively. DIM did not show significant affinity for the ER in the range of concentrations used.

We studied the binding of the ER to its cognate DNA motif (ERE) by a gel mobility shift assay using nuclear extracts from MCF-7 cells treated with E_2 or DIM. A 31-mer 5′-^{32}P-labeled double-stranded oligonucleotide containing the consensus ERE motif was used as the probe. Figure 4 shows that there was a clearly shifted band for the ERE from nuclear extracts of cells treated with E_2 or DIM, and that this band shift was obvious at a DIM concentration of only 1 μM. Pretreatment of these nuclear extracts with an anti-ER monoclonal antibody produced the supershifted band expected for the antibody–ER–ERE complex. These results demonstrate that DIM is capable of activating ER binding to the ERE by a mechanism that does not

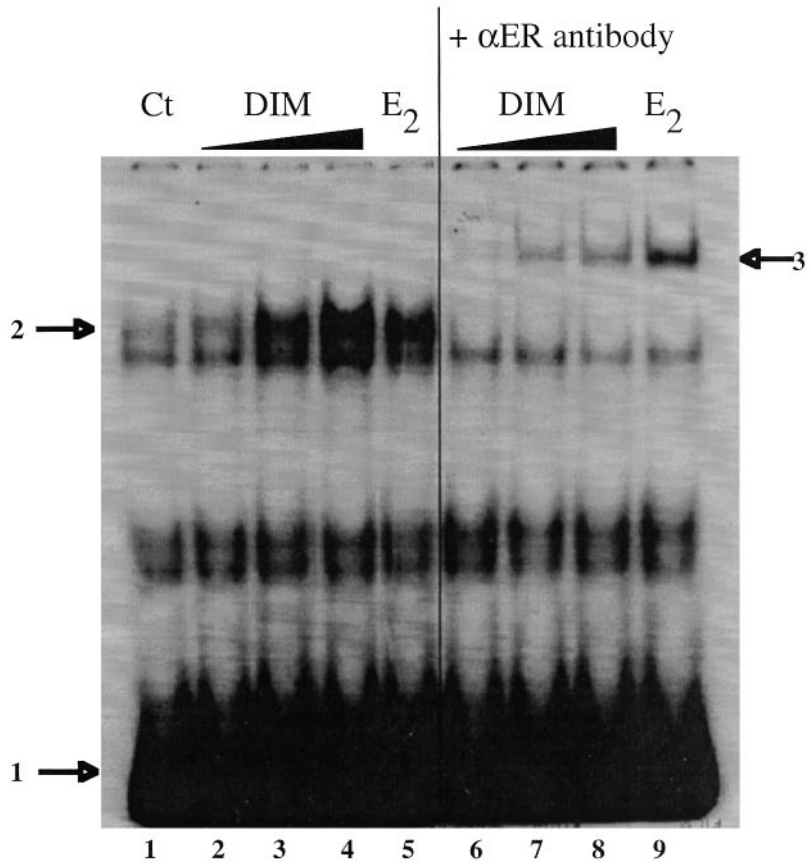

FIG. 4. Binding of nuclear proteins to the ERE. Gel mobility shift analysis of nuclear extracts from estrogen-depleted MCF-7 cells treated for 2 hr with DMSO (lane 1, control), DIM (0.1, 1.0, and 10.0 μM in lanes 2 and 6, 3 and 7, and 4 and 8, respectively), and E_2 (1 nM) (lanes 5 and 9). A monoclonal antibody specific for the human ER was also added to the incubation mixture for lanes 6–9. Arrows indicate the locations of the free labeled probe (arrow # 1), the ligand-responsive shifted band (arrow # 2), and the antibody supershifted band (arrow # 3).

require the binding of DIM to the ligand-binding domain of the ER.

Promoter Specificity of DIM Activation of ER-Responsive Genes and Sensitivity to a Standard Antiestrogen

To assess the effects of DIM on estrogen-responsive gene expression, MCF-7 cells were treated with DIM in the presence or absence of E_2 for 2 days, and northern blots were probed for E_2-responsive pS2 gene expression (Fig. 5). We found that when added to E_2-depleted medium, DIM induced the expression of endogenous pS2 mRNA in a concentration-dependent manner. However, co-treatment of cells with E_2 and DIM produced no statistically significant effect on the level of pS2 mRNA expression over that produced by E_2 treatment alone.

To examine whether the effects of DIM on E_2-responsive genes were affected by promoter conformation, cells were transfected transiently with the ERE-containing CAT-reporter plasmids pERE-vit-CAT or pS2-tk-CAT. DIM stimulated CAT activity of both reporters when administered in the absence of E_2 and weakly reduced E_2-induced expression of both of these reporters (Fig. 6). Thus, DIM functioned at the level of transcriptional activation of these two complex promoters. We did not observe the agonist effects of DIM, however, when cells were transfected with the simpler ERE-CAT reporter, pATC2, which was responsive to E_2 and to the synthetic estrogen DES (Fig. 7). Nevertheless, the weak antagonist effect of DIM on E_2-induced expression was also seen with this construct. These observations indicate that the agonist effects of DIM are restricted to a subset of E_2-responsive constructs.

Effects of Antiestrogens and Overexpression of ER on the Agonist Effects of DIM

Next, we examined whether the ER is essential to the agonist effects of DIM. First, we determined the effects of the pure antiestrogen ICI on DIM-induced activation of the pERE-vit-CAT reporter. The results presented in Fig. 8 show that ICI inhibited the activities of both DIM and E_2, and that ICI was a more effective inhibitor of DIM-induced activity than of E_2-induced activity. Whereas ICI produced a 90% reduction in E_2-induced activity, the combination of

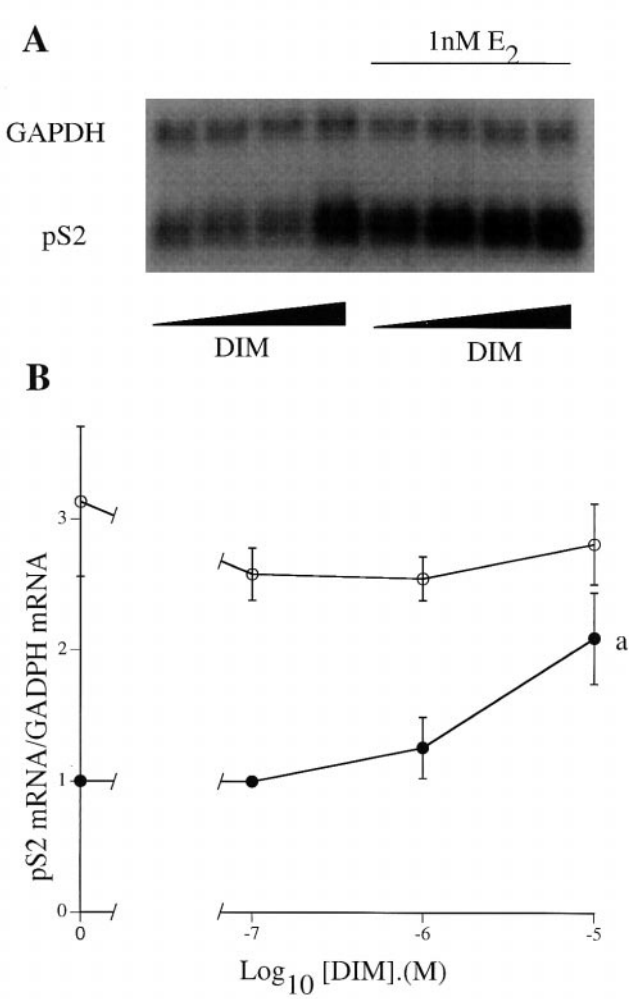

FIG. 5. Effect of DIM on pS2 mRNA expression. Estrogen-depleted MCF-7 cells were treated for 48 hr with DIM at concentrations ranging from 0.1 to 10.0 μM, with (○) or without (●) E_2 (1 nM). pS2 mRNA levels were measured by northern-blot analysis and normalized using GADPH mRNA as an internal standard. (A) Typical autoradiograph. (B) Results are presented as fold induction over the DMSO control, as the means ± SD for three separate experiments. Key: (a) significantly different ($P < 0.05$) from the DMSO control.

DIM and ICI reduced the activity to below the control values and resulted in greater than a 100% reduction of DIM-induced activity. These relative effects of E_2, DIM, and ICI on pERE-vit-CAT expression were unchanged by high levels of ER produced by co-transfection of MCF-7 cells with the pCMV-hER constitutive ER expression vector (data not shown). Taken together, these results provide further evidence that the ER plays a key role in the agonist effects of DIM.

Ligand-Independent Transcriptional Activation in MCF-7

To begin to examine the mode of ligand-independent activation of ER by DIM, we verified that ligand-independent transcriptional activation of the ER could be demonstrated with the ERE-vit-CAT reporter assay. Transfected cells were treated with a specific D1-dopamine receptor agonist, SKF-82958, shown previously to have that activity

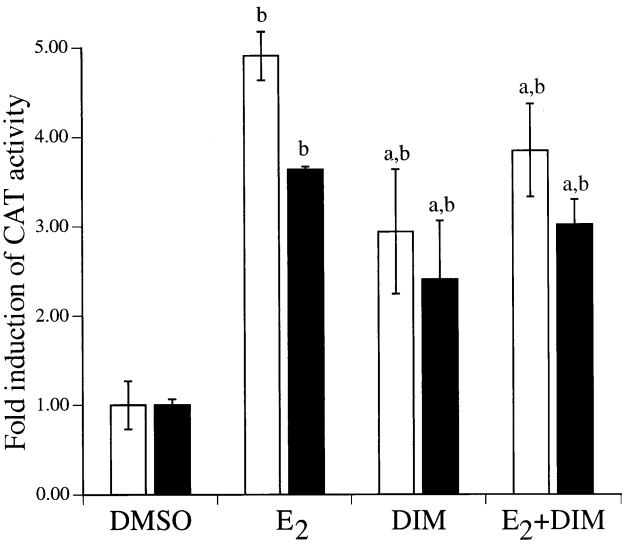

FIG. 6. Effect of DIM on CAT expression from the ERE-vit-CAT and pS2-tk-CAT reporters. MCF-7 cells were transfected transiently with the ERE-vit-CAT (black bars) or the pS2-tk-CAT (white bars) reporter plasmid and treated for 48 hr with vehicle control, E_2 (1 nM), DIM (10 μM), or a combination of the two. CAT activity in cytosol preparations from individual plates was normalized for protein concentration. Data are presented as fold induction over the DMSO control (means ± SD of four independent determinations). Key: (a) significantly reduced ($P < 0.05$) from E_2-induced; and (b) significantly different ($P < 0.05$) from the DMSO control.

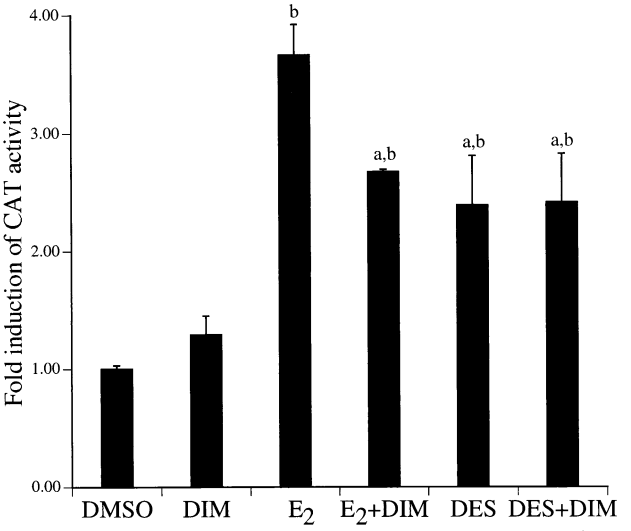

FIG. 7. Effect of DIM on CAT expression from the pATC2 reporter. MCF-7 cells were transiently transfected with the pATC2 reporter plasmid and treated for 48 hr with vehicle control, E_2 (1 nM), DES (1 nM), DIM (10.0 μM), or in combinations of E_2 or DES with DIM. Data are presented as fold induction over the DMSO control (means ± SD of four independent determinations). Key: (a) significantly reduced ($P < 0.05$) from E_2-induced, and (b) significantly different ($P < 0.05$) from the DMSO control.

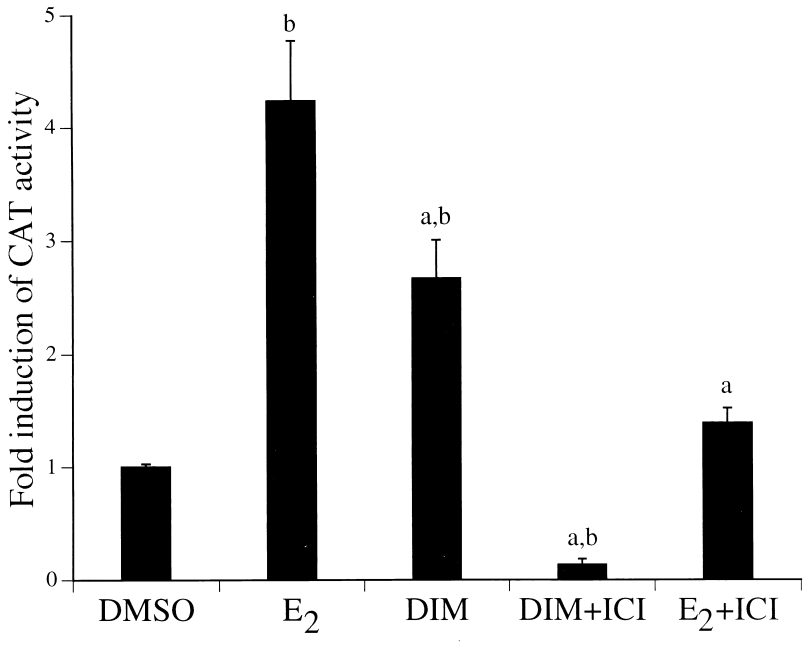

FIG. 8. Effect of antiestrogen on DIM- or E_2-induced CAT expression from the ERE-vit-CAT reporter. MCF-7 cells transiently transfected with the ERE-vit-CAT reporter plasmid were treated for 48 hr with DIM (10.0 μM) or E_2 (1 nM), with or without ICI (0.1 μM). Data are presented as fold induction over the DMSO control (means ± SD of four independent determinations). Key: (a) significantly reduced ($P < 0.05$) from E_2-induced; and (b) significantly different ($P < 0.05$) from the DMSO control.

in a neuroblastoma-derived cell line [26]. Figure 9 shows that 10 μM SKF-82958 induced transcription of the reporter gene to the same level as did E_2. This dopaminergic effect on ER function was blocked by the PKA inhibitor H-89. Importantly, H-89 completely ablated the response to DIM but exhibited no effect on the response to E_2, clearly indicating different modes of ER activation by the two substances. These results suggest that DIM-mediated activation of the ER may involve a PKA activation pathway.

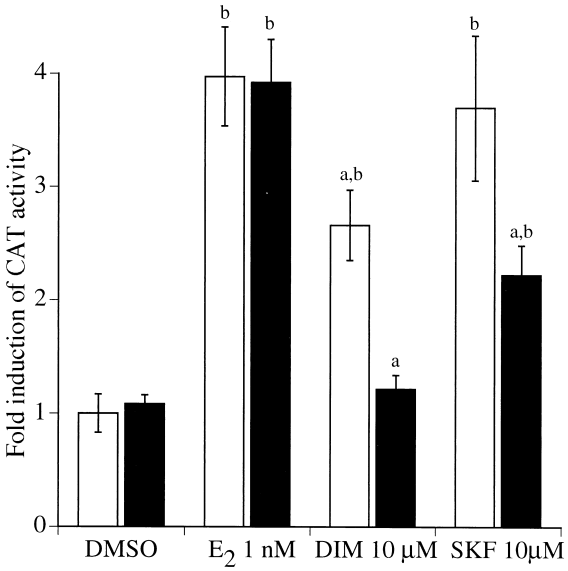

FIG. 9. Ligand-independent activation of transcription of an ER-responsive reporter gene. MCF-7 cells transiently transfected with the ERE-vit-CAT reporter plasmid were treated for 48 hr with DIM (10.0 μM), E_2 (1 nM), or SKF-82958 (10.0 μM) with (black bars) or without (white bars) the PKA inhibitor H-89 (10.0 μM). Data are presented as fold induction over the DMSO control (means ± SD of four independent determinations). Key: (a) significantly reduced ($P < 0.05$) from E_2-induced; and (b) significantly different ($P < 0.05$) from the DMSO control.

DISCUSSION

Our results show that at concentrations of up to 10 μM, DIM exhibited primarily agonist activity on cell proliferation and transcriptional activation of E_2-responsive endogenous and transfected reporter genes. Although DIM did not interact with the ligand-binding site of the ER, DIM activated the ER to a form that binds *in vitro* to the consensus ERE. In the absence of E_2, DIM promoted a level of cell proliferation in the E_2-responsive MCF-7 cell line that was approximately 60% of the maximum rate produced by E_2, but reduced the maximum E_2-induced proliferation rate of this cell line by about 40%. At the concentrations effective against proliferation of MCF-7 cells, however, DIM had very little effect on the proliferation of the E_2-independent cell line MDA-MB-231. Thus, under the conditions used in this study, the proliferative effects of DIM were cell type-specific and may require interaction with the ER.

In addition to the effects of DIM on cell proliferation, DIM could also function as an activator of E_2-responsive reporter genes and endogenous gene expression. In the absence of E_2, DIM exhibited pronounced agonist activities

with E_2-responsive genes regulated by complex promoters (ERE-vit-CAT, pS2-tk-CAT, endogenous pS2). DIM, however, had little effect on activation of a basal E_2-responsive reporter construct, pATC2. DIM did not affect significantly the E_2-induced expression of the endogenous pS2 gene and was a weak antagonist of E_2-induced expression of all three reporter constructs. The agonist effects of DIM were efficiently blocked by the antiestrogen ICI. ICI, considered a pure antiestrogen, functions specifically by binding to and inactivating the ER. These results indicate that the agonist effects of DIM require a pathway that involves both the activation of the ER and the presence of a *cis*-acting DNA element in addition to the consensus ERE or the core promoter. The consistently weak but generally significant antagonist effects of DIM on E_2 functions in E_2-responsive breast tumor cells are less dependent on promoter context and thus, may be important in long-term cancer prophylaxis.

The results emphasize the potential importance of an interaction of I3C-derived indoles on E_2-activated cellular processes, independent of possible effects of indoles on E_2 metabolism. Several studies have appeared suggesting that a major antiestrogenic mode of action of dietary indoles is via the metabolic deactivation of E_2 by an indole-induced increase in the activities of certain cytochrome P450 oxidative enzymes [27]. DIM, on the contrary, has been shown to competitively inhibit the activities associated with CYP1A1, CYP1A2, and CYP2B1 enzymes, and is an antagonist of AhR-mediated gene activation [19]. Our observation of the ER agonist effects of DIM strongly suggests that DIM can interact directly with the ER pathway and that the tumor growth inhibitory effects of I3C products may arise from effects in addition to or independent of increased E_2 metabolism.

There has been considerable recent concern over the presence of hormonally active contaminants in the environment [28]. These so-called endocrine disrupters have been suggested to be involved in various hormone-related abnormalities including decreased sperm count, birth defects, and breast cancers. To adequately assess the relative significance of such substances, it is important to consider their activities relative to the natural background of such activities produced by plant products including DIM and other hormonally active substances in the diet. Agonist activities on estrogen-dependent growth of breast tumor cells also have been reported for several other phytochemicals including certain flavonoids, such as genistein and apigenin, and lignins such as enterolactone [29]. As a result, the total dose of natural, estrogenically active substances from the diet can be substantial. For example, the amount of I3C in a 100-g portion of broccoli is about 50 mg, which would be expected to provide between 5 and 10 mg of DIM following ingestion and acid-catalyzed conversion in the stomach. The total dose of estrogenically active substances from a typical Western diet is estimated to be in excess of 1 g/day, which, when estrogenic potency is considered, is far in excess of the estrogen equivalent dose from environmental contaminants [29].

The results presented here are an interesting extension to results of related studies that report antiproliferative and apoptotic effects of DIM [30]. In a recent study by Chen *et al.* [31] DIM was reported to behave as an antagonist of estrogen function: at concentrations below 10 μM, DIM strongly inhibited the proliferation of a variant of cultured MCF-7 cells selected for their sensitivity to TCDD. At higher concentrations, DIM inhibited E_2-induced expression of a vitellogenin A2 gene promoter–CAT reporter construct co-transfected with an ER expression plasmid into MCF-7 cells. Also, in contrast with our results, these investigators reported that in gel mobility shift analyses of extracts of cells cultured in complete medium, the higher concentrations of DIM decreased the intensity of the ER–ERE bands. We observed strong antiproliferative effects on E_2-responsive MCF-7 cells and in E_2-nonresponsive MDA-MB-231 cells at DIM concentrations above 10 μM. However, since treatment with these concentrations was accompanied by considerable cell death and possible induction of apoptosis, we restricted the present studies to the lower concentrations. It is interesting to note that the proliferative effects of DIM reported in the aryl hydrocarbon-nonresponsive MCF-7BaPr cells [30] are similar to the effects we observed in our wild-type estrogen-responsive MCF-7 cells, i.e. DIM-induced proliferation in the absence of E_2 and weak inhibition of E_2-induced proliferation. Since the AhR signal transduction pathway is functional in our wild-type cells, as shown by the ICZ-induced expression of EROD activity (data not shown), it appears that the reported low concentration antagonistic effects of DIM are dependent on factors other than or in addition to a functional AhR.

The importance of the promoter- and cell-specific agonist effects of DIM reported here to the well-established protective effects of I3C against carcinogen-induced mammary cancer is yet to be defined. An overall picture is emerging, however, suggesting that the biological effects of oral I3C and of the major acid products of I3C are primarily estrogenic. We reported previously on the activities of two other major acid condensation products of I3C, namely the linear trimeric product, LTr-1, and the cyclic trimeric product, CTr. We observed that LTr-1 exhibited weak ER antagonist activities in the breast tumor cells that required concentrations in excess of 10 μM [32]. In contrast, we observed that CTr was a potent ligand for the ER and exhibited strong ER agonist activities at concentrations as low as 0.1 μM [33]. An important indication of the combined *in vivo* effects of the I3C acid products was reported recently by Oganesian *et al.* [34]. These investigators showed that oral I3C produces clearly estrogenic effects in trout. It is possible, therefore, that I3C may function in a manner similar to that suggested for the anticarcinogenic isoflavone genistein. Hsieh *et al.* [35] reported that genistein is a strong ER agonist and at physiological concentrations produces an estrogenic stimulation of mam-

mary development and enhanced proliferation of implanted MCF-7 cells in rodents. Lamartiniere *et al.* [36] and Hsieh *et al.* [35] suggest that the cancer protective effects of estrogen agonists such as genistein may result from effects on the maturation of the mammary gland. It is possible that the protective effects of I3C and DIM may arise from a similar estrogenic mechanism.

The case for the agonist effect of DIM occurring through a ligand-independent activation of the ER is strongly supported by the fact that DIM has no affinity for the ligand binding site but causes ER to bind to its cognate DNA motif and to activate transcription of E_2 responsive genes. The fact that this transcriptional activation was totally blocked by the specific ER antagonist ICI establishes the central role of the ER in this process. Our observation that the PKA inhibitor H-89 blocked the DIM-induced activity of the ERE-vit-CAT reporter but had no effect on the E_2-mediated activation of this reporter suggests a role for PKA in the observed agonist activities of DIM. Several reports of ligand-independent activation of the ER have appeared and show that the human ER responds to certain growth factors, phosphatase inhibitors, kinase activators, and neurotransmitters [26]. Whether DIM triggers similar physiologically important pathways and whether these pathways are involved in the cancer protective effects of DIM are important objectives of our ongoing research in this area.

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