Indole-3-Carbinol Selectively Uncouples Expression and Activity of Estrogen Receptor Subtypes in Human Breast Cancer Cells

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Estrogen-responsive breast cancer cells, such as MCF7 and T47D cells, express both estrogen receptor (ER)-α (ERα) and ERβ. Indole-3-carbinol (I3C) strongly down-regulated ERα protein and transcript levels, without altering the level of ERβ protein, in both cell lines. In cells transfected with the ERα promoter linked to a luciferase gene reporter, I3C ablated ERα promoter activity. Propyl pyrazole triol (PPT) is a highly selective ERα agonist, whereas, 17β-estradiol activates both ERα and ERβ. I3C treatment inhibited the PPT- and 17β-estradiol-induced proliferation of breast cancer cells, disrupted the PPT and 17β-estradiol stimulation of estrogen response element (ERE)-driven reporter plasmid activity as well as of endogenous progesterone receptor transcripts. Using an in vitro ERE binding assay, I3C was shown to inhibit the level of functional ERα and stimulated the level of ERE binding ERβ even though the protein levels of this receptor remained constant. In ERα-/-/ERβ+ MDA-MB-231 breast cancer cells, I3C treatment stimulated a 6-fold increase in binding of ERβ to the ERE. I3C also induced ERE- and activator protein 1-driven reporter plasmid activities in the absence of an ER agonist, suggesting that ERβ is activated in indole-treated cells. Taken together, our results demonstrate that the expression and function of ERα and ERβ can be uncoupled by I3C with a key cellular consequence being a significantly higher ERβ:ERα ratio that is generally highly associated with antiproliferative status of human breast cancer cells. (Molecular Endocrinology 20:3070–3082, 2006)

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STROGENS ARE A class of steroid hormones that play a critical role in the development of the normal breast and in the genesis of breast cancer (1, 2). Estrogens mediate their cellular responses through two distinct intracellular receptors, estrogen receptor (ER)-α (ERα) and ERβ, which are encoded by separate genes (3). In the cell systems tested to date, after activation by ligand binding, each ER regulates the transcription of unique but overlapping sets of target genes (4), although both receptors are capable of activating synthetic reporter plasmids driven by estrogen response elements (EREs) or activator protein 1 (AP-1) sites. The two ERs have been shown to play different roles in the reproductive physiology of mammals (5, 6). For example, a high ERα:ERβ ratio correlates well with high levels of cellular proliferation, whereas predominance of functional ERβ over ERα correlates with lower levels of proliferation (7–9). Ectopic expression of ERα in estrogen-sensitive breast cancer cell lines, such as MCF7 and T47D, leads to no major changes in proliferation rate, whereas expression of exogenous ERβ in these cells leads to a drastic reduction in cell growth rates in culture as well as in nude mouse xenografts (10, 11). This correlation with the proliferative state also holds in a physiological setting in that during the pre and peri-pubertal phases of rodents, which are periods of high proliferation, ERα expression predominates in the mammary epithelium, whereas, during lactation, in which there is minimal mammary epithelial mitogenesis, the amount of ERβ protein is 5 times that of ERα in the mammary epithelium (12, 13). Additionally, in the ERα null mouse mammary gland, even pharmacological doses of estrogen are ineffective in inducing robust growth of mammary epithelium, indicating that ERβ does not mediate proliferative effects of estrogens in the mammary epithelium (2).

Although the role of ERβ in the etiology of human breast cancer has not been well characterized, ERα expression and function appears to be an important factor for prognosis of human breast cancer. ERα-positive breast cancers correlate well with the proliferation of differentiated tumor phenotypes, and the majority of these tumors respond well to antihormonal therapy (14). Tamoxifen, a mixed antiestrogen, is clinically useful in the management of ERα-positive met-

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Abbreviations: AP-1, Activator protein 1; CDK, cyclin-dependent kinase; CMV, cytomegalovirus; DIM, 3’-3’-indolylmethane; DMSO, dimethyl sulfoxide; E, 17β-Estradiol; ER, estrogen receptor; ERE, estrogen response element; GAPDH, Glyceraldehyde phosphate dehydrogenase; I3C, Indole-3-carbinol; PPT, Propyl pyrazole triol; RLU, relative light unit; SDS, sodium dodecyl sulfate.
astatic breast cancer and in the prevention of breast cancer, and has improved the survival of women with ERα-positive breast cancer (15, 16). However, long-term management of breast cancer with tamoxifen is associated with tamoxifen resistance, increased endometrial cancer risk, and other undesirable side effects (15–17). Tamoxifen-resistant breast cancers respond well to the pure steroidal antiestrogen ICI 182,780 (Faslodex), which has been shown to down-regulate the levels of ERα by causing proteasome-dependent degradation (18–21). Thus, a key issue in the characterization of promising therapeutic molecules to treat breast cancers is to determine their specific effects on ERα and ERβ responsiveness.

Both epidemiological and physiological studies suggest that phytochemicals from vegetables and fruits represent a largely untapped source of potential anti-cancer molecules (22–27). One such phytochemical is indole-3-carbinol (I3C), an autolysis product of glucosinolates present in Brassica vegetables such as broccoli, cabbage, and cauliflower. Dietary exposure to I3C reduced tumor occurrence and decreased the multiplicity of spontaneous as well as carcinogen-induced mammary tumor formation in rodent model systems (28, 29). I3C also tested positive as a chemopreventative agent in several short-term bioassays relevant to carcinogen-induced DNA damage, tumor initiation and promotion, and oxidative stress (30). We and others have demonstrated that I3C and its natural dimer, 3′-3′-dindolylmethane (DIM) have direct anti-proliferative effects on human reproductive cancer cells (31–35). For example, I3C induces a G1 cell cycle arrest of both estrogen-sensitive MCF7 and estrogen-insensitive MDA-MB-231 breast cancer cells (36) that is accompanied by a transcriptional down-regulation of cyclin-dependent kinase (CDK) 6 (39), and an inhibition of CDK2 activity in which the size distribution of associated cyclin E forms and subcellular localization of the CDK2 protein complex is altered (37). I3C also cooperates with tamoxifen to more effectively induce a G1 cell cycle arrest and ablated phosphorylation of the retinoblastoma protein (Rb) in MCF7 breast cancer cells (32). This indole has also been shown to elicit antiestrogenic effects and decrease the level of phosphorylated ERβ protein (38), although the underlying mechanism was not examined.

In this study, we report that at concentrations of I3C effective in causing a G1 cell cycle arrest, I3C differentially regulates the expression and function of ERα and ERβ. This indole strongly down-regulated ERα expression due to a loss of promoter activity, which disrupts the regulated expression of exogenous and endogenous genes by 17β-estradiol (E) and by propyl pyrazole triol (PPT), a highly selective agonist for ERα, without altering ERβ expression. Furthermore, we report that the loss of ERα expression is accompanied by an increase in functional ERβ that can bind to an ERE and increase ERβ-associated ERE and AP-1-driven transcriptional activity. Thus, our study demonstrates that treatment with I3C results in a significantly higher ERβ:ERα ratio of functional ER subtypes that is highly associated with antiproliferative status of human breast cancer cells.

RESULTS

I3C Down-Regulates ERα Protein Levels without Altering ERβ Protein Levels in MCF7 and T47D Human Breast Cancer Cells

Estrogen-responsive breast cancer cells express two ER subtypes (ERα and ERβ) that are encoded by distinct genes (3). ERα responsiveness is generally associated with proliferative mechanisms, whereas dominance of ERβ responsiveness has been linked to antiproliferative processes (2, 3, 11, 12). To examine the relative expression levels of ERα and ERβ under I3C-mediated growth-arrested conditions (36), estrogen-responsive MCF7 and T47D human breast cancer cells were treated with various doses of I3C for 48 h, and Western blots of total cell extracts were probed for either ERα or ERβ protein. The levels of β-actin were used as a gel loading control. As shown in Fig. 1A, I3C strongly down-regulated the level of ERα protein in both cell lines in a dose-responsive manner, without causing any effect on the level of ERβ protein. Western blots showed that 1 μM E was unable to reverse the I3C-mediated down-regulation of ERα protein (data not shown), suggesting that I3C does not act as an ER antagonist in agreement with our previous report demonstrating that I3C does not bind ERs (32). Figure 1B (top panel) shows the quantification by densitometric measurement of ERα and ERβ protein levels in cells treated at various I3C concentrations in comparison to the G1 cell cycle arrest induced by I3C in MCF7 cells as determined by flow cytometry. The overall dose-dependent profile of down-regulation of ERα protein levels strongly correlated with that of the increase in G1 phase arrested cells and down-regulation of S phase cells. The half-maximal indole responses were approximately 100 μM I3C, and the maximal responses without any apoptotic effects were observed at 200 μM I3C. The data shown in Fig. 1B are for MCF-7 cells, although the same effects were observed with T47D human breast cancer cells (data not shown). Taken together, these data show that the maximal indole concentrations, which induce a G1 cell cycle arrest, result in a predominantly ERβ-responsive cellular background, and therefore 200 μM I3C was the dose used throughout the remainder of this study.

To determine the kinetics of I3C induced down-regulation of ERα protein, MCF7 and T47D cells were treated with or without 200 μM I3C for different durations, and total cell extracts were examined for the levels of ERα and ERβ protein, as well as that of CDK2 protein as a loading control (36), by Western blot analysis. As shown in Fig. 2, the I3C-mediated down-regulation of ERα protein is a rapid response that occurs within the first 24 h of indole treatment in both...
Fig. 1. Dose-Dependent Effects of I3C Production of ERα and ERβ Protein in MCF7 and T47D Human Breast Cancer Cells

A, MCF7 and T47D cells were treated with the indicated concentrations of I3C for 48 h. Isolated protein extracts were fractionated using SDS-PAGE and electrophoretically transferred to nitrocellulose membranes, and immunoblots were probed with antibodies specific for ERα and ERβ. β-Actin protein was used as a gel loading control. The band intensities were quantified using densitometry, and the bar graph represents relative values for ERα and ERβ normalized to actin values.

B, MCF7 cells were exposed to DMSO or indicated doses of I3C for 48 h, hypotonically lysed in a solution containing propidium iodide, and subjected to flow cytometric analysis based on DNA content. This analysis is depicted as a bar graph with SE bars.
though only the data using MCF7 cells are shown. Results were obtained for MCF7 and T47D cells, all cancer cells. In all of the following experiments, similar gnucleotide primers. Expression of ER examined by RT-PCR analysis using gene-specific oligonucleotides, and immunoblot analysis for ERα and ERβ was carried out as described in Materials and Methods. CDK2 protein levels have been shown to remain unaltered with I3C treatment and were used as a protein loading control.

cell lines and is maintained throughout the time course. The results with T47D cells show that I3C down-regulated ERα protein levels without altering ERβ levels within 6 h of I3C treatment. Although the earliest time point shown for MCF7 cells is 24 h, I3C down-regulated ERα protein production with a similar overall kinetic profile as observed in T47D cells. The levels of CDK2 remained unaltered throughout each time course as expected. Thus, our results show for the first time that an antiproliferative phytochemical can selectively down-regulate the production of the ERα without altering the level of ERβ in human breast cancer cells. In all of the following experiments, similar results were obtained for MCF7 and T47D cells, although only the data using MCF7 cells are shown.

I3C Selectively Down-Regulates ERα Transcript Levels and Promoter Activity

The relatively rapid down-regulation of ERα protein by I3C suggested that this indole might be selectively regulating the transcription of this ER subtype. To test whether I3C down-regulated ERα mRNA levels, MCF7 cells were treated with dimethylsulfoxide (DMSO) as the vehicle control, 200 μM I3C, 1 μM tamoxifen, an antiestrogen known to cause G1 cell cycle arrest in MCF7 cells (17, 32), or 200 μM tryptophol for 48 h. Tryptophol is structurally similar to I3C, differing by one carbon, but fails to induce a growth arrest (39) and therefore can be used as an indole structural control. Total RNA was isolated and the levels of ERα and ERβ transcripts were examined by RT-PCR analysis using gene-specific oligonucleotide primers. Expression of ERα and ERβ transcripts were compared with glyceraldehyde phosphate dehydrogenase (GAPDH), which is constitutively expressed. As shown in Fig. 3, I3C treatment abrogated the expression of ERα mRNA without any effect on ERβ mRNA levels. Exposure to either tamoxifen or tryptophol had no effect on ERα or ERβ transcript levels, demonstrating that the I3C down-regulation of ERα transcripts is not a fortuitous consequence of the growth arrest or a nonspecific indole effect.

The ERα gene promoter has been shown to have two transcriptional start sites (denoted A and B), although precise function and regulation of each site have not been completely characterized. Sp1 transcription factor binding sites have been shown to be critical for expression of ERα (40). I3C has been shown to transcriptionally down-regulate CDK6 by disrupting Sp1 binding to consensus binding sites (39). To test whether I3C regulates ERα promoter activity, MCF7 cells were transfected with two different ERα promoter luciferase reporter plasmids. One reporter plasmid contains 3.2 kb of the ERα promoter (−3200-luc) with both transcriptional start sites, and the other construct contains a 0.7-kb fragment of the ERα promoter (−700-luc) with only transcriptional start site A (see diagram in Fig. 4A, bottom panel). Transfected cells were treated with or without I3C for 24 h, and the luciferase-specific activity was monitored in both sets of transfected cells. Two controls routinely used in these experiments are transfection of the empty pGL2 vector as well as transfection of a construct expressing the luciferase reporter under the constitutive cytomegalovirus (CMV) promoter. I3C did not affect the production of luciferase in either control (data not shown). As shown in Fig. 4A, I3C treatment strongly down-regulated the promoter activity [measured in relative light units (RLU)] of the −3200-luc reporter gene, which contains both ERα transcriptional start sites. In contrast, I3C had no effect on activity of the −700 ERα promoter fragment, which has been observed to have significant activity when transfected in MCF7 cells. Both plasmids show considerable luciferase activity in untreated MCF7 cells upon transfection in that in the absence of I3C the −3200-Luc reporter plasmid displayed approximately 1020 RLU per μg protein, and the −700-Luc reporter plasmid displayed approximately 800 RLU per μg protein. Thus, the loss of I3C down-regulation of promoter activity from the −700-Luc reporter plasmid reflects the loss of a putative
I3C-regulated element rather than simply an inactive promoter fragment. These results demonstrate that the I3C down-regulation of ER
 gene products can be accounted for by a concomitant down-regulation of ER promoter activity. Consistent with this promoter effect, I3C failed to strongly ablate mRNA levels of ER in MCF7 cells transfected with plasmids containing ER cDNA for ectopic overexpression. Control and ER overexpressing MCF7 cells were treated with DMSO or 200 μM I3C for 48 h. Total RNA isolated from these cells was subjected to reverse transcription followed by PCR with primers specific for ER or GAPDH. PCR products were electrophoresed and visualized using a UV transilluminator.

**I3C Disrupts ER-Responsive ERE Activity and Estrogen-Induced Expression of Endogenous Progesterone Receptor**

To examine the functional consequences of I3C treatment on ER responsiveness, MCF7 cells were grown in steroid-deficient media supplemented with 10% charcoal dextran-stripped fetal bovine serum, transfected with a pGL2 luciferase reporter plasmid containing the consensus vitellogenin ERE (Vit-3-ERE-luc). In this assay, I3C was tested for its effectiveness in disrupting ERE-driven luciferase expression induced by E, or by the ER-specific agonist PPT. PPT has been shown in several systems to be highly ER specific, possessing 410-fold higher affinity for ER than for ERβ (4). Transfection of the empty pGL2 luciferase vector provided a negative control for background luciferase activity. The cells were pretreated with DMSO or 200 μM I3C for 24 h, and then incubated with the indicated combinations of DMSO, 200 μM I3C, 10 nM E, and 100 nM PPT for an additional 24 h. ERE
activity was measured in cell extracts as RLUs and normalized to total protein. As shown in Fig. 5, treatment with either E or PPT stimulated a robust increase in ERE activity. Incubation with I3C ablated the stimulated ERE activity by both ER agonists (I + E; I + PPT). Treatment with I3C alone induced a small but reproducible increase in ERE and AP-1 reporter plasmid activities, which as described later is likely due to activation of ERβ.

To assess whether I3C can disrupt the regulated expression of an endogenous estrogen-responsive gene, MCF7 cells were treated with combinations of 200 μM I3C and either 10 nM E or 100 nM PPT. Cells were cultured in steroid-deficient media supplemented with 10% charcoal dextran-stripped fetal bovine serum. Using specific oligonucleotide primers, the expression of progesterone receptor transcripts, an estrogen target gene, was examined in comparison to GAPDH, which is constitutively expressed. As shown in Fig. 6, the robust E and PPT stimulation of PR transcripts is strikingly ablated in the presence of I3C. This result demonstrates that I3C down-regulation of ERα has functional consequences in that it results in disruption of total activity at a given ERE, as well as in ablation of E or PPT to stimulate expression of ERα-dependent genes such as progesterone receptor.

**Differential Effects of I3C on the Binding of ERα and ERβ to the ERE**

Given that I3C can disrupt ERE-driven transcriptional activity as well as induction of estrogen responsive genes in MCF7 cells, an *in vitro* binding assay was employed to evaluate effects of I3C treatment on the ability of ERα or ERβ to bind to an ERE. Streptavidin–agarose beads were conjugated with biotinylated oligonucleotides encoding the ERE from the vitellogenin promoter, and then incubated with cell lysates isolated from I3C-treated and untreated MCF7 or MDA-MB-231 breast cancer cells which are ERα+/ERβ−. ERE bound proteins were eluted in a high-salt buffer, electrophoretically fractionated in sodium dodecyl sulfate (SDS) polyacrylamide gels, and immunoblotted for ERα or ERβ using subtype-specific antibodies. As shown in Fig. 7A (*left panel*), I3C down-regulated the level of ERE-bound ERα protein in MCF7 cells, which is accounted for by the I3C down-regulation of total ERα protein levels (Fig. 7B, *left panel*). Strikingly, even though the total level of ERβ protein did not change (Fig. 7B, *right panel*) after indole treatment, I3C significantly increased the level of ERβ that can bind to the ERE (Fig. 7A, *right panel*). Quantification of these results demonstrated that I3C reverses the ratio of functional ERβ to ERα in MCF7 cells, effectively leading to a primarily ERβ-responsive cellular background.
To assess I3C regulation of ERβ activity in breast cancer cells, MDA-MB-231 cells were transfected with luciferase reporter plasmids containing ERE reporter constructs. As shown in Fig. 7A (left panel), I3C treatment caused a 6-fold increase in ERβ binding to the ERE, whereas ERα did not bind to the ERE under these conditions. The MDA-MB-231 cells do not express ERα in the presence or absence of I3C (Fig. 7B, right panel), and therefore DNA binding was not assessed for this ER subtype.

I3C Treatment Increases ERβ-Mediated ERE and AP-1 Activity in MDA-MB-231 Breast Cancer Cells

To assess I3C regulation of ERβ activity in cells expressing ERβ in the absence of ERα, MDA-MB-231 cells were transfected with luciferase reporter plasmids containing highly estrogen-responsive DNA elements, Vit-3×ERE or AP-1. Cells were then cultured in media supplemented with steroid-deficient fetal bovine serum in the presence of the indicated combinations of 10 nM E and 200 μM I3C for 24 h, and relative luciferase-specific activity was measured as described in Materials and Methods. A parallel experiment was performed with 1 μM tamoxifen instead of E. As shown in Fig. 8A, I3C treatment caused a reproducible activation of the ERE-containing reporter plasmid activity (DMSO vs. I3C) in the absence or presence of 17β-estradiol. As expected, E had no effect on ER activity in this ERα-deficient cell line. As shown in Fig. 8B, I3C treatment also increased AP-1-driven luciferase activity independent of E treatment. As shown in Fig. 8C, the robust I3C-mediated increase in AP-1 activity was partially reversed by tamoxifen, in accordance with previous reports that suggest that I3C enhances ERβ responsiveness even in the absence of an ER agonist.

I3C Treatment Disrupts Estrogen-Induced Proliferation of MCF7 Breast Cancer Cells

The growth of estrogen-responsive human breast cancer cell lines, such as MCF7 and T47D cells, is strongly stimulated by estrogens when cultured in steroid-deficient conditions (17). Therefore, MCF7 cells grown in steroid-deficient media containing 10% charcoal dextran-stripped fetal bovine serum were employed to test whether I3C can block cell proliferation induced by either E or PPT. Cell cycle effects were examined by flow cytometry of propidium iodide-stained nuclei after 48 h in culture in the indicated conditions. As shown in Fig. 9A (left panels), treatment with either 10 nM E or 100 nM PPT induces cell-cycle progression in both agonists strongly reduced the number of cells in the G1 phase of the cell cycle (~66% to ~46%) and stimulated a significant increase in S phase cells (~29% to ~50%). Exposure to 100 μM I3C for 24 h blocked the stimulation in S phase cells and decrease in G1 phase cells by either E or by the ERα-specific agonist PPT (Fig. 9A, right panels). In the steroid-free conditions of this experiment, the cells remained relatively growth arrested in the presence or absence of I3C, and the overall DNA content was not altered (Fig. 9A, top panels). The cell cycle effects observed in three independent experiments were quantified, and as shown in Fig. 9B, the overall distribution of DNA content of cells treated with a combination of I3C and either E or PPT was nearly identical to that of cells not exposed to any estrogens. Similar results were also observed using human T47D breast cancer cells (data not shown). Thus, key cellular consequences of the selective I3C disruption of ERα expression are loss of total cellular ERα activity and estrogen-dependent proliferation.
DISCUSSION

Human estrogen-responsive breast cancer cell lines that are highly proliferative, such as MCF7 and T47D, express high levels of ERα compared with normal mammary epithelial cells and other breast cancer cells. Overexpression of exogenous ERβ in these cell lines has been shown to lead to a marked arrest in growth and disruption of 17β-estradiol-induced proliferation (10, 11). An emerging concept that developed...
I3C treatment also caused a reproducible en-
cent cells in G1, S, and G2 phases with SE bars.

dent experiments were quantified.

Methods.

B, The flow cytometry results from three indepen-
to flow cytometry analysis as described in

Supplemental Methods. B, The flow cytometry results from three indepen-
dent experiments were quantified. Bar graph indicates per-
cent cells in G1, S, and G2 phases with SE bars.

Fig. 9. I3C Suppresses E and ERα-Specific Agonist PPT-
Induced Proliferation of MCF7 Breast Cancer Cells

A, MCF7 cells were grown in steroid-deficient media sup-
plemented with 10% dextran charcoal-stripped fetal bovine
serum. Cells were pretreated with DMSO or 100 μM I3C for
24 h, and then treated with DMSO or 100 μM I3C (I) in the
presence or absence of 10 nM E or 100 nM PPT for 24 h. Cells
were harvested in PBS and stained with a hypotonic solution
containing propidium iodide. Stained nuclei were subjected

from these and other studies is that an increased
ERα:ERβ ratio in estrogen-responsive mammary cancer
cells is conducive to cell growth whereas an in-
creased ERβ:ERα ratio is generally linked to antipro-
liferative contexts (7–9, 41). In the present study using
both MCF7 and T47D breast cancer cell lines, we
demonstrate that the I3C growth arrest is accompa-
nied by a selective down-regulation of ERα expression
and responsiveness, while ERβ levels remain unal-
tered. I3C treatment also caused a reproducible en-
hancement of functional ERβ as monitored by its bind-
ing to the vitellogenin ERE. Thus, I3C reversed the
functional ER ratio to primarily an ERβ-responsive
background, which correlated well with the potent an-
tiproliferative effects of this indole. Thus, a novel re-
sponse of I3C is the selective transcriptional down-
regulation of ERα expression without altering protein
or transcript levels of ERβ.

Strong correlation has been found between cellular
proliferation in normal mammary glands and mam-
mary cancers to the relative levels of ERα and ERβ.
ERβ expression levels were higher in normal human
mammary epithelial cells \textit{in vivo}, and in nonprolifera-
tive benign breast disease compared with its levels in
more malignant preinvasive lesions, such as ductal
carcinoma \textit{in situ}, which usually overexpress ERα (7, 8, 14). The roles of ERα and ERβ in mammary epithelial
cell proliferation have been evaluated in knock out
mouse models (2, 3), as well as in human breast can-
cer cell lines in culture (10, 11). The proliferative effects
of estrogens are thought to be mediated primarily by
ERα, because ablation of ERα in mouse mammary
glands leads to a severely underdeveloped mammary
epithelium. The ERα null epithelium still expresses
ERβ, and the mammary epithelial growth impairment
cannot be rescued by administration of pharmacological
levels of estrogens (2). This genotype is also as-
associated with a major decrease in the development of
estrogen-unresponsive mammary cancers indicating
that estrogen signaling through ERα plays a major role
in proliferation of normal mammary epithelial cells \textit{in vivo} as well as in hormone-responsive and hormone-
unresponsive mammary carcinogenesis (1, 2). Current
breast cancer therapeutic drugs that inactivate ERα,
such as tamoxifen, have been shown to prevent de-
velopment of carcinogen-induced rat mammary tu-
mors (16) and prevent breast cancer in humans (42),
which further establishes a positive correlation be-
tween ERα and cellular proliferation as well as mam-
mary carcinogenesis. In contrast to the information
currently available on ERα, the precise significance of
ERβ in human breast cancers remains unclear, al-
though ERβ is thought to play a role in inhibiting mam-
mary epithelial cell proliferation during lactation and
involution in the normal mammary gland. ERβ null
mice show normal development of the mammary
gland until lactation. Lactating wild-type mice show a
marked cessation of mammary epithelial cell prolifer-
ation, whereas ERβ null mice show significant levels
of mammary epithelial cell proliferation during lactation
(43).

Our observation that I3C down-regulates ERα gene
expression by an inhibition of promoter activity is con-
sistent with the results of our previous studies dem-
strating that I3C strongly down-regulates CDK6
promoter activity in human breast cancer cells that
undergo a G1 cell cycle arrest (36, 39). I3C disrupts the
function of the Sp1 and ETS transcription factors at a
composite element in the CDK6 promoter (31, 39).
Interestingly, the kinetics of ERα down-regulation by
I3C is strikingly similar to that of CDK6 (39), suggesting
that an analogous regulatory mechanism may be operating on ERα. Consistent with this concept, I3C inhibited a luciferase reporter plasmid containing a 3200-bp ERα promoter fragment, but had no effect when the reporter plasmid was linked to a 700-bp 5′-deleted promoter fragment. The larger ERα promoter fragment contained both the transcription start sites (B at −1994 bp and A), suggesting the existence of an I3C-regulated transcriptional element in the ERα promoter between −3200 and −700. This region contains binding sites for Sp1, the binding of which is disrupted by I3C leading to transcriptional down-regulation of CDK6 (39). We are currently in the process of determining the precise location of the I3C-regulated element and of identifying which transcription factor(s) binding is/are targeted by I3C.

Several lines of evidence show that a key consequence of the I3C down-regulation of ERα transcription is the loss of ERα responsiveness. I3C ablates the stimulation of growth and activation of an ERE reporter plasmid by PPT, a highly selective ERα agonist that possess a 400-fold higher affinity for ERα. Furthermore, I3C treatment blocks the E and PPT-stimulated expression of progesterone receptor, a known ERα-responsive gene. Interestingly, PPT stimulates growth of MCF7 and T47D cells to the same extent as 17β-estradiol. I3C also appears to activate ERβ by inducing a significant increase in the DNA binding form of ERβ, and by stimulating vitellogenin ERE reporter plasmids in MDA-MB-231 breast cancer cells that express ERβ, but not ERα. It is interesting to note that I3C is effective in causing a cell cycle arrest in MDA-MB-231 breast cancer cells (36), and this correlates well with activation of ERβ. These breast cancer cells are unlikely to express any unusual ERα isoforms because this gene is heavily silenced by methylation (40). We are currently attempting to elucidate the mechanism by which I3C activates ERβ function in human breast cancer cells.

Estrogen signaling has been shown to be essential for the proliferation of normal mammary epithelial cells and is a key risk factor in the development of estrogen-responsive as well as estrogen-unresponsive breast cancer (1, 3, 14, 17, 42). It has been established that benign lesions showing elevated levels of ERα pose a higher risk for development of malignant breast cancer linking ERα to estrogen-mediated increase in tumorigenicity in rodents and humans (44, 45). Conceivably, a potential prophylactic use of I3C is to down-regulate ERα in high-risk but benign ERα overexpressing premalignant cell populations, thus effectively dampening the promotion of premalignant lesions to malignancy by estrogens. Furthermore, I3C has been shown to cooperate with the antiestrogen tamoxifen in that a combination of tamoxifen and I3C caused a more potent growth arrest than either compound alone (32). These findings suggest that this indole could be used as a potential adjuvant therapeutic for estrogen-responsive breast cancer. Taken together, our results implicate I3C as a new class of therapeutic molecules that mediate their anticancer effects in estrogen-responsive cell types through the selective control of ER gene expression.

MATERIALS AND METHODS

Materials

DMEM, Iscove’s modified Dulbecco’s medium, fetal bovine serum, calcium- and magnesium-free PBS, l-glutamine, and trypsin-verumine mixtures were purchased from BioWhittaker, Inc. (Walkersville, MD). Insulin (bovine), and 17β-estradiol, dimethyl sulfoxide, and tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO). PPT was obtained from Tocris (Ellisville, MO). I3C, DIM, and Tryptophol were purchased from Aldrich (Milwaukee, WI). The sources of other reagents are either listed below or were of the highest purity available.

Cell Culture

MCF7 and T47D human breast adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). MCF7 cells were grown in DMEM supplemented with 10% fetal bovine serum (BioWhittaker), 10 μg/ml insulin, 50 U/ml penicillin, 50 U/ml streptomycin, and 2 mM l-glutamine. T47D cells were grown in Iscove’s modified Dulbecco’s medium containing 10% fetal bovine serum, 2 mM l-glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin. The cells were grown to subconfluence in a humidified air chamber at 37 C containing 5% CO2. Stock solutions of I3C, DIM, Tryptophol (99.9% HPLC grade), 17β-estradiol, PPT, and tamoxifen were dissolved in DMSO to yield appropriate concentrations. DMSO was used as vehicle control for all experiments.

ERα Gene Promoter Transfections and Luciferase Activity Assays

Plasmids containing 5′-deletions of ERα gene promoter were a kind gift from Dr. Lisa McPherson, Stanford University. MCF7 cells, grown to confluency in six-well plates, were transfected with 1 μg of plasmid constructs containing either −3.2 kb upstream region of the ERα gene with both promoter sites B and A or a 700-bp upstream region containing only start site A. Transfection was performed in serum-supplemented media using FuGene 6 (Roche, Indianapolis, IN) as per manufacturer’s instructions. Cells were treated 24 h post transfection with DMSO or 200 nM I3C for 48 h. Cells were lysed, and relative luciferase activity was evaluated using Promega luciferase assay kit (Promega Corp., Madison, WI) and a luminometer. Relative luciferase activities were normalized to the protein input with SE. Reproducibility of these results was verified with three independent experiments performed with triplicate samples for each treatment.

ERE-Luciferase/AP-1-Luciferase Transfections and Luciferase Assays

Plasmid (1 μg) containing the consensus vitellogenin vit-3×ERE in pgll2 vector or the consensus AP-1 sites was transfected according to manufacturer’s instructions using FuGene 6 (Roche). The media were replaced, 24 h later, with media containing 10% dextran charcoal-stripped fetal bovine serum, and then after an additional 24 h, the cells were treated with DMSO or 200 nM I3C for 48 h. The cells were then treated with DMSO, 10 nM 17β-estradiol, or 100 nM PPT.
Western Blotting for ERα and ERβ Protein

After the indicated treatments, MCF7 and T47D cells were harvested in the media, pelleted by centrifugation at 1000 rpm for 5 min, resuspended in 1 ml PBS, and pelleted again by centrifugation. These cells were then resuspended in radio immunoprecipitation buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, and 0.4% SDS (pH 6.8)], and fractionated on 8% polyacrylamide/0.1% SDS resolving gels using electrophoresis. Proteins were then transferred electrically to nitrocellulose membranes for 1 h at 4 C. Equal loading was confirmed with Ponceau S incubation of membranes. The blots were then blocked for 1 h in 5% nonfat dry milk dissolved in Western wash buffer [10 ml Tris·HCl (pH 8), 150 mM NaCl, and 0.05% Tween 20] at room temperature. The blots were the rinsed in Western wash buffer and incubated overnight at 4 C in antibodies diluted in Western wash buffer. Rabbit anti-ERα and -ERβ antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz CA) were diluted 1:1000 in wash buffer. CDK2 immunoblotting was performed as previously described (34). Immunoreactive proteins were detected after incubation with horseradish peroxidase-conjugated secondary antibodies diluted to 3 × 10−4 in 1% non-fat dry milk in Western wash buffer (goat antirabbit IgG; Bio-Rad Laboratories). Blots were then treated with enhanced chemiluminescence reagents (NEN Life Sciences, Boston, MA) and all proteins were detected by exposure to Hyperfilm (Eastman Kodak, Rochester, NY). Immunoblotting for ERα and ERβ was repeated using total protein extracts from two independent experiments.

RNA Extraction and RT-PCRs

MCF7 cells were grown in appropriate media and, after the indicated treatment durations, were harvested in 1 ml of Trizol reagent (Invitrogen, San Diego, CA), and the recommended protocol was followed to extract total RNA. RNA was quantified using spectroscopy, and the quality of RNA was confirmed using A260/A280, and by electrophoresis on 1% agarose gels. Total RNA (2 μg) was subjected to reverse transcription using Mu-MLV Reverse Transcriptase (Invitrogen) with random hexamers, deoxynucleotide triphosphates, and RNase Inhibitor (Invitrogen). cDNA (4 μl) was then subjected to PCR using primers specific to ERα, ERβ, PR, and GAPDH as described elsewhere (46) The PCR products were electrophoretically fractionated in 1% Agarose gels along with a 1-kb plus DNA ladder and visualized on a UV transilluminator. RT-PCR was then performed on total RNA from two independent experiments to confirm the observed expression patterns for ERα and ERβ.

Affinity Chromatography for ER-ERE Binding

The pBluescript plasmid containing the consensus vitellogenin ERE was subjected to restriction enzyme digest using BglII and HindIII. The products were electrophoresed on a 1% agarose gel containing ethidium bromide. The 550-bp band corresponding to the ERE was excised and extracted using the Qiaex II kit (QIAGEN, Chatsworth, CA). Approximately 16 μg of ERE fragment was biotinylated using Klenow enzyme as per manufacturer’s protocol and was extracted by ethanol/salt extraction. Overall, 7 μg of DNA was then used for the chromatographic assay. MCF7 and MDA-MB-231 cells treated with I3C or DMSO were lysed using a buffer containing 10 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, and 2 mM EDTA, and a cocktail of protease inhibitors without dithiothreitol. Protein content of cell lysates was then normalized using the Lowry method (Bio-Rad Laboratories). Streptavidin-conjugated agarose beads (Sigma) were resuspended in 400 μl of lysis buffer, and the column was divided into two Eppendorf tubes with 200 μl each. Approximately 2 mg of total protein from DMSO- or I3C-treated cells was added to each column and incubated at 4 C for 1 h with constant rocking using a nutator (Clay Adams, Sparks, MD). The column was then centrifuged at 4 C for 5 min at 5000 rpm. The unbound lysate was stored, and the columns were washed three times using lysis buffer. The elution buffer was prepared by adding 400 mM NaCl to the lysis buffer and added to the beads. After incubation at room temperature for 5 min, the beads were centrifuged, and the supernatant was loaded and subjected to SDS-PAGE. The amount of bound ERs was assayed by immunoblotting as described above. Using different batches of cells and drugs, this procedure was repeated five independent times.

Flow Cytometric Analyses of DNA Content

MCF7 cells were plated onto six-well tissue culture dishes (Nunc Fisher, Hampton, NH). The cells were grown in media containing dextran charcoal-stripped 10% fetal bovine serum. Cells were treated with 10 nM E or 100 nM PPT in the presence and absence of 100 μM I3C. Cells were incubated for 48 h and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, and 0.05% Triton X-100). Nuclear emitted fluorescence with wavelength of more than 585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Nuclei (10,000) were analyzed from each sample at a rate of 300–500 nuclei. The percentages of cells within the G1, S, and G2–M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley. This experiment was repeated twice to confirm observed results.

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