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# Estrogen receptor α as a target for indole-3-carbinol

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**Abstract**

A wealth of preclinical evidence supports the antitumorigenic properties of indole-3-carbinol (I3C), which is a major bioactive food component in cruciferous vegetables. However, the underlying molecular mechanism(s) accounting for these effects remain unresolved. In the present study, estrogen receptor alpha (ER-α) was identified as a potential molecular target for I3C. Treating MCF-7 cells with 100 μM I3C reduced ER-α mRNA expression by approximately 60% compared to controls. This reduction in ER-α transcript levels was confirmed using real-time polymerase chain reaction. The I3C dimer, 3,3′-diindolylmethane (DIM), was considerably more effective in depressing ER-α mRNA in MCF-7 cells than the monomeric unit. The suppressive effects of 5 μM DIM on ER-α mRNA was comparable to that caused by 100 μM I3C. DIM is known to accumulate in the nucleus and is a preferred ligand for aryl hydrocarbon receptor (AhR) to I3C. The addition of other AhR ligands, α-naphthoflavone (α-NF, 10 μM) and luteolin (10 μM), to the culture media resulted in a similar suppression in ER-α mRNA levels to that caused by 5 μM DIM. Thus, it is likely that the binding of ligands to AhR inhibits nuclear ER-α transcript. The results from these experiments suggest that the antitumorigenic effects of I3C in MCF-7 human breast cancer cells may arise from its ability to reduce ER-α expression through the binding of its metabolite, DIM, to the nuclear AhR.


*Keywords:* Indole-3-carbinol; 3,3′-Diindolylmethane; Estrogen receptor α

## 1. Introduction

Breast cancer influenced by genetic and/or environmental factors is one of the most prevalent cancers in the United States. Fluctuation in estrogen exposure is considered one of the important risk factors in the development of cancer at this site [1]. Many of estrogen's biological activities are triggered by binding of estrogen to its receptors including ER-α [2]. ER-α is a ligand-activated transcription factor and a member of the nuclear receptor superfamily [3]. ER-α is involved not only with the proliferative but also with the antiapoptotic effects of estrogen [4]. Using an ER-α knockout mouse model, Bocchinfuso et al. [5] demonstrated the critical role of ER-α in estrogen-dependent promotion of mammary tumor growth. Thus, it is likely that ER-α plays a significant role in breast cancer prevention and/or therapy [6].

Indole-3-carbinol (I3C) is a phytochemical that has been documented in numerous epidemiological and preclinical studies to possess mammary cancer preventive properties [7,8]. Glucobrassicin, which is found in a variety of cruciferous vegetables including broccoli, Brussel's sprout, cabbage, cauliflower and kale, is the primary source of I3C in the human diet. Once I3C reaches the acidity of the stomach, it can be converted to a number of derivatives [9]. One of the most prevalent derivatives found in humans is 3,3′-diindolylmethane (DIM) [10]. Mounting preclinical evidence with cell cultures and animal models suggest that both I3C and DIM have promising effects on breast cancer prevention [11–13]. However, it remains unclear how these interact with specific targets among the pathways involved with estrogen. The current studies were designed to examine ER-α as a key target for dietary indoles.

*Abbreviations:* ER-α, Estrogen receptor alpha; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; I3C, Indole-3-carbinol; DIM, 3,3′-Diindolylmethane; RT-PCR, Reverse transcriptase-mediated polymerase chain reaction.

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## 2. Materials and methods

### 2.1. Reagents and cell lines

Microarray analyses were performed using the 3DNA Submicro EX Expression Array Detection kits that were purchased from Genisphere (Hatfield, PA). The cDNA microarray slides (Hs-UniGEM2) containing 10,368 genes were obtained from the Advanced Technology Center, National Cancer Institute (Gaithersburg, MD). Human mammary cancer cell line MCF-7 was obtained from American Type Culture Collection. Media and reagents for cell culture and Trizol were from Invitrogen (Carlsbad, CA). I3C (purity, 29%) was purchased from Sigma (St. Louis, MO) and used without additional purification. DIM was purchased from LKT laboratories (St. Paul, MN). Qiagen RNeasy Midi Kit was purchased from Qiagen (Valencia, CA). Alpha-naphthoflavone (α-NF) and luteolin were purchased from Sigma (St. Louis, MO).

### 2.2. Cell culture

MCF-7 cells were cultured as described previously [4]. The experiments were conducted in media containing 5% charcoal-dextran-treated serum to minimize background steroid levels. For microarray experiments, $1\times10^8$ cells/flask were plated into T-175 flasks. Twenty-four hours after plating, cells were treated with either 100 μM I3C or vehicle (dimethylsulfoxide). For reverse transcriptase-mediated polymerase chain reaction (RT-PCR) experiments, $1\times10^6$ to $2\times10^6$ cells were plated on 6-well plates. Twenty-four hours after plating, cells were treated with either vehicle or test compounds. In all experiments, media containing either test compounds or vehicle were replenished daily. In experiments using the aryl hydrocarbon receptor (AhR) antagonists, cells were treated with vehicle, 100 μM I3C or 10 μM DIM for 4 h prior to the addition of α-NF (10 μM) or luteolin (10 μM) and allowed to grow for a total of 72 h.

### 2.3. Cell number determination

For experiments examining time-dependent effects of I3C or DIM on MCF-7 cells, cells were treated in the presence or absence of I3C (100 μM) or DIM (25 μM) for 0–72 h. Cells ($1\times10^5$ cells/well) were plated in 24-well plates, and treatments began 24 h after plating. To assess effects of I3C or DIM on estradiol, cells were treated with or without $10^{-10}$ M 17β-estradiol in the presence or absence of I3C (100 μM) or DIM (5 μM). We used lower DIM concentrations to avoid complications from cell death. Cell number was assessed using a previously published method [14].

### 2.4. RNA isolation

Total RNA for microarray studies was isolated using a combination of Trizol and Qiagen RNeasy Midi Kit methods. Briefly, cells were lysed directly in a T-175 flask with 5 ml Trizol (Invitrogen). The lysate was transferred into a 50-ml Falcon screw cap tube (Becton Dickinson, Franklin Lakes, NJ), and chloroform (200 μl/ml Trizol) was added. The tubes were then vortexed for 20 s and centrifuged at 4°C, 12,000×*g* for 15 min. The clear top layer containing the RNA was recovered, and an equal volume of 70% ethanol was added. Samples were than mixed and loaded on to Qiagen RNeasy Midi Kit. Purification continued from this point following Qiagen's protocols. For other experiments, total RNA was isolated as described previously [14].

### 2.5. Probe synthesis and detection of differential gene expression using cDNA microarray

Differential expression of genes between control and treated cells was assessed using cDNA microarray techniques. The Genisphere 3DNA detection methods (3DNA Submicro EX Expression Array Detection kit, Genisphere, Hatfield, PA) were used for labeling and detection of differential expression. Briefly, total RNA from control and treated cells was reverse transcribed using RT primers tagged with either Cy5- (control) or Cy3- (treated) specific 3DNA capture sequence. After hybridization of the probes to cDNA microarray slides, the synthesized tagged cDNAs were then fluorescent labeled by Cy3-3DNA or Cy5-3DNA based on the complementary capture sequence of 3DNA capture reagents. After stringency washes, the microarray slides were then scanned using ScanArray 4000 microarray scanner (Perkin-Elmer, Boston, MA). The intensity of Cy3- and Cy5-labeled spots was quantified using QuantArray software (Perkin-Elmer). The results are expressed as a relative value to the control.

### 2.6. Determination of estrogen receptor alpha mRNA levels

Semiquantitative RT-PCR was used to validate changes in estrogen receptor alpha (ER-α) mRNA levels. RT was performed using 2 μg of total RNA as previously described [4]. The TaqMan real-time PCR method was then used to quantify ER-α message levels. TaqMan reactions were carried out using the TaqMan universal PCR master mix (Applied Biosystems, Branchburg, NJ) in a total volume of 25 μl on an ABI-PRISM 7000 Sequence Detector (Applied Biosystems). Oligonucleotide primers and TaqMan probe for ER-α mRNA were made according to published sequences [15]. Primers and a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene were purchased from ABI-PE Applied Biosystems. For the determination of ER-α mRNA, 200 nM probe and 300 nM of each primer were used. For GAPDH, 100 nM probe and 40 nM of each primer were used. The amplifications were performed in triplicate for each sample, and the PCR optimal conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The Ct method, as described in the manufacturer's protocol, was used to generate relative expression value.

### 2.7. Statistics

Experimental data were analyzed using the Statview statistical software package (SAS Institute). Paired *t* tests were used for two group comparisons. For multiple group comparisons, ANOVA followed by post hoc analysis using Fisher's test were employed. Treatments with a *P* value of <.05 were considered significantly different.

## 3. Results

### 3.1. Gene array analysis

A cDNA microarray technique was used to profile differentially expressed genes in the ER-positive MCF-7 human breast cancer cells. The supplementation with the concentration of 100 μM I3C did not alter cell morphology or viability. The NCI human array chips containing about 10,000 genes were hybridized with probes prepared either from the control or 100 μM I3C-treated MCF-7 cells to screen targets for I3C. When only one chip was analyzed, the result showed about 300 genes up-regulated and 200 down-regulated (data not shown). When the same experiment was repeated three times independently, the number of genes altered was considerably reduced and the difference was consistently significant. The average of the three independent experiments of the top 20 up-regulated genes is listed in Table 1 and down-regulated genes are listed in Table 2. T/C indicates the ratio of treated cells over the control. In response to 100 μM I3C, GJA8 gap junction protein was increased by approximately 100%. One hundred micromolars of I3C supplementation also up-

Table 1
Top 20 up-regulated genes from three microarray analysis of mRNA from MCF-7 cells

| Name | Mean (T/C) | S.D. | *P* (paired *t*) |
|---|---|---|---|
| GJA8 (gap junction protein, alpha 8, 5) | 2.10 | 0.213 | .012 |
| NMA (putative transmembrane protein) | 1.83 | 0.189 | .017 |
| ARVCF (armadillo repeat gene deletes in velocardiofacial syndrome) | 1.83 | 0.266 | .033 |
| MMP23B (matrix metalloproteinase 23B) | 1.80 | 0.193 | .019 |
| MGC33993 (hypothetical protein MGC33993) | 1.75 | 0.156 | .014 |
| ESTs (moderately similar to hypothetic RED protein) | 1.73 | 0.139 | .012 |
| MDK (midkine; neurite growth promoting factor 2) | 1.71 | 0.140 | .013 |
| PCDH17 (protocadherin 17) | 1.71 | 0.222 | .031 |
| KIAA0819 (KIAA0819 protein) | 1.61 | 0.241 | .048 |
| FLJ36888 (hypothetical protein FLJ3688) | 1.60 | 0.172 | .027 |
| DPEP1 (dipeptidase 1; renal) | 1.57 | 0.141 | .020 |
| C1QTNF2 (C1q and tumor necrosis factor-related protein 2) | 1.56 | 0.181 | .033 |
| ESTs | 1.56 | 0.182 | .034 |
| BRAP (BRCA1-associated protein) | 1.55 | 0.146 | .023 |
| COCH (coagulation factor C homolog, cochlin [Limulus polyphemus]) | 1.55 | 0.197 | .041 |
| EST | 1.54 | 0.125 | .017 |
| NOS1 (nitric oxide synthase 1[neuronal]) | 1.53 | 0.201 | .045 |
| BLu (BLu protein) | 1.52 | 0.207 | .049 |
| FLJ13611 (hypothetical protein FLJ1361) | 1.51 | 0.201 | .048 |
| CAP2 (adenylyl cyclase-associated protein) | 1.50 | 0.153 | .029 |

T = with 100 μM I3C; C = without 100 μM I3C.

Table 2
Top 20 down-regulated genes from three microarray analysis of mRNA from MCF-7 cells (T: with, C: without 100 μM I3C)

| Name | Mean (T/C) | S.D. | *P* (paired t) |
|---|---|---|---|
| CDC45L (CDC45 cell division cycle 45-L) | 0.654 | 0.098 | .026 |
| AK1 (adenylate kinase 1) | 0.653 | 0.129 | .043 |
| BAG1 (BCL2-associated athanogene) | 0.647 | 0.043 | .005 |
| EBAG9 (estrogen receptor binding site) | 0.646 | 0.107 | .029 |
| AGR2 (anterior gradient 2 homolog [Xenopus laevist]) | 0.635 | 0.112 | .030 |
| ARG2 (arginase, type II) | 0.634 | 0.093 | .021 |
| ENO2 (enolase 2; gamma, neuronal) | 0.633 | 0.127 | .038 |
| ACVRL1 (activin receptor kinase 1-like) | 0.624 | 0.066 | .010 |
| DAP3 (death association protein 3) | 0.616 | 0.135 | .039 |
| DKFZp586H062 (putative UDP-GalNAc:polypeptide N-acetygalactosaminyltransferase) | 0.601 | 0.130 | .033 |
| AMPD1 (adenosine monophosphate deaminase 1 [isoform M]) | 0.573 | 0.143 | .035 |
| CUTL1 (cut-like 1, CCAAT displacement) | 0.573 | 0.070 | .009 |
| B4GALT6 (UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6) | 0.570 | 0.088 | .014 |
| CLK2 (CDC-like kinase 2) | 0.558 | 0.070 | .008 |
| ESPL1 (extra spindle poles like 1 [S. cerevisiae]) | 0.532 | 0.044 | .003 |
| DNAJC3 (DnaJ [Hsp40] homolog, subfamily C member 3) | 0.488 | 0.078 | .008 |
| ERCC6 (excision repair cross-complementing rodent repair deficiency, complimentation group 5) | 0.455 | 0.210 | .046 |
| ALOX5AP (arachidonate 5-lipoxygenase-activating protein) | 0.451 | 0.123 | .016 |
| ESR1 (estrogen receptor 1) | 0.415 | 0.027 | .001 |
| BMP7 (bone morphogenetic protein 7) | 0.309 | 0.156 | .017 |

T = with 100 μM I3C; C = without 100 μM I3C.

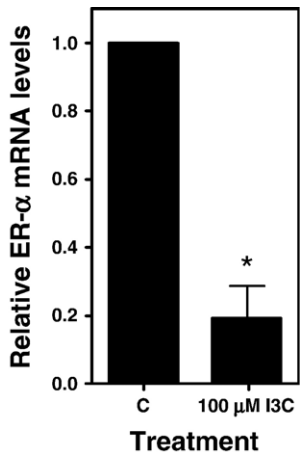


Fig. 1. Effects of 100 μM I3C on ER-α mRNA levels in MCF-7 cells analyzed by real-time RT-PCR analysis. MCF-7 cells were treated with and without I3C, and total RNA was isolated as described in Material and methods. Taqman real time PCR method was used to determined ER-α mRNA levels as described in the Materials and methods. Results are expressed as mean±S.D. ($n=3$). *Significantly different from control at $P<.05$.

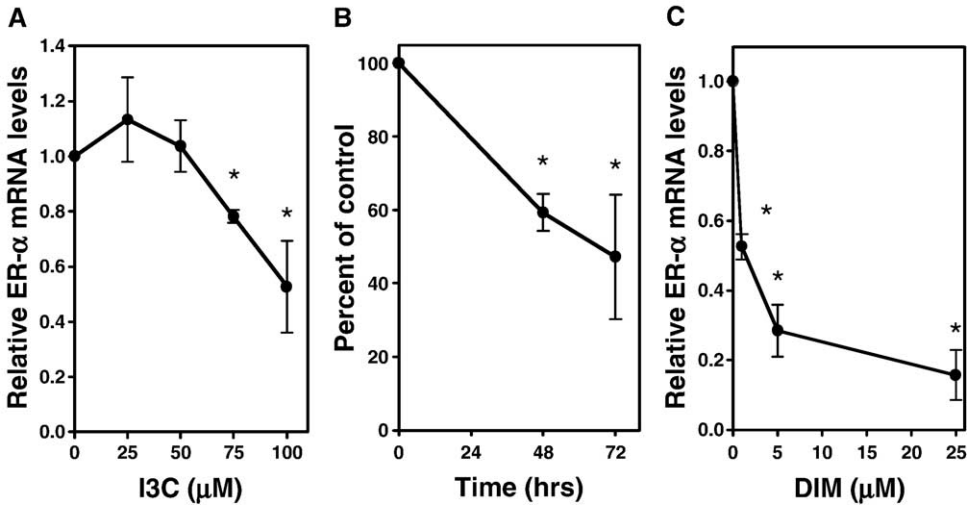


Fig. 2. Effects of I3C and DIM on ER-α mRNA levels. (A) Concentration-dependent effects. MCF-7 cells were treated with 0–100 μM I3C for 72 h and total RNA isolated as described in Materials and methods. Real-time RT-PCR analysis using the Taqman real-time PCR methods was performed to determine ER-α mRNA levels as described in the Material and methods. Results are expressed as mean±S.D. ($n=3$). (B) Time-dependent effects. MCF-7 cells were treated with and without 100 μM I3C for 0–72 h and RNA isolated as described in Material and methods. Real-time RT-PCR analysis using the Taqman real-time PCR methods was used to determine ER-α mRNA levels as described in Material and methods. Results are expressed as mean±S.D. ($n=3$). *Significantly different from control at $P<.05$. (C) Effects of DIM on ER-α mRNA levels in MCF-7 cells. MCF-7 cells were treated with 0–25 μM DIM for 72 h and total RNA isolated as described in Materials and methods. Real-time RT-PCR analysis using the Taqman real-time PCR methods was performed to determine ER-α mRNA levels as described in the Materials and methods. Results are expressed as mean±S.D. ($n=3$). *Significantly different from control at $P<.05$.

regulated a number of other genes including matrix metalloproteinase 23B (MMP23B), BRCA1-associated protein (BRAP) and nitric oxide synthase 1 (NOS1). The down-regulation of ER-α is consistent with the previous report by Ashok et al. [11].

### 3.2. ER-α is an I3C responsive gene in MCF-7 cells

The gene designated as ESR1, more commonly known as ER-α, surfaced as one of the most markedly affected genes (Table 2). One hundred micromolars I3C reduced ER-α mRNA by approximately 60% compared to controls. To confirm this change, additional experiments were performed using a semiquantitative real-time RT-PCR. This method again revealed that ER-α mRNA levels were substantially decreased (Fig. 1, $P<.001$).

### 3.3. Characterization of the effect of I3C on ER-α mRNA levels in MCF-7 cells

The dose dependency and temporal effects of I3C in ER-α were also examined. As shown in Fig. 2A, the effects of I3C were concentration-dependent. More than 50 μM I3C was required to reduce ER-α mRNA levels in the MCF-7 cell (Fig. 2A). Temporally, the difference in ER-α mRNA levels was observed after 48-h treatment with 100 μM I3C (Fig. 2B), and the suppressed message levels were maintained during the following 24-h incubation period.

### 3.4. DIM down-regulates ER-α mRNA levels in MCF-7 cells

Others have reported that treatment of MCF-7 cells with I3C can lead to an accumulation of the condensation product DIM in the nucleus, suggesting a role for DIM in transcription [16,17]. Hence, DIM was also evaluated for its ability to alter ER-α mRNA levels. Addition of DIM at concentrations greater than 25 μM was cytotoxic and prevented the recovery of RNA for analysis (data not shown). ER-α mRNA in MCF-7 cells treated with 0–25 μM

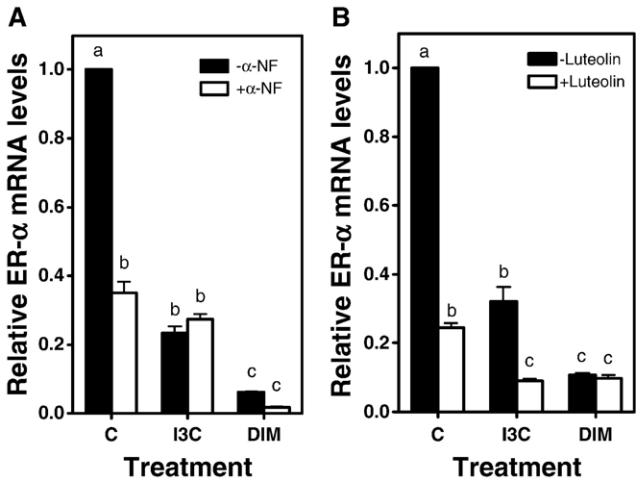


Fig. 3. Effects of I3C and DIM on ER-α mRNA expression in the presence or absence of AhR antagonists, α-NF and luteolin, in MCF-7 cells. (A) MCF-7 cells were treated with 100 μM I3C or 10 μM DIM in the presence or absence of 10 μM α-NF for 72 h. (B) MCF-7 cells were treated with 100 μM I3C or 10 μM DIM in the presence or absence of 10 μM luteolin for 72 h. Total RNA was isolated as described in Material and methods. Real-time RT-PCR analysis using the Taqman real-time PCR methods was performed to determine ER-α mRNA levels as described in Materials and methods. Results are expressed as mean±S.D. ($n=3$). Bars with different letters were significantly different at $P\leq.05$.

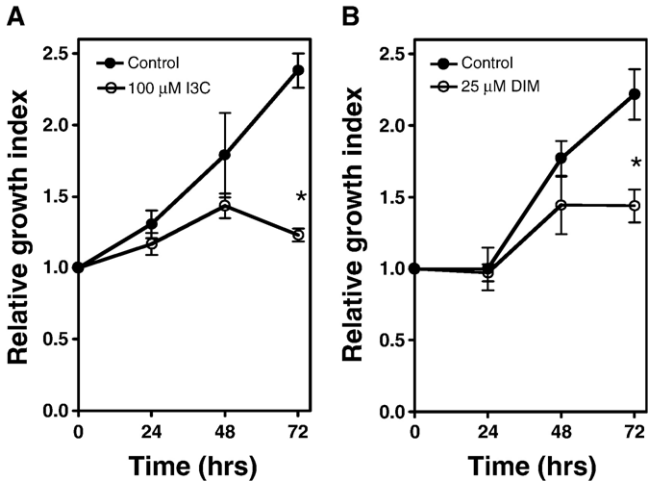


Fig. 4. Effects of I3C (A) and DIM (B) on MCF-7 cell number. To assess effects of I3C and DIM on MCF-7 cells, cells were treated with vehicle (control), 100 μM I3C, or 25 μM DIM for 0–72 h, and cell numbers were determined as described in Material and Methods. Results are expressed as mean±S.D. ($n=4$). *Significantly different form control at $P<.05$.

DIM (Fig. 2C) lowered ER-α mRNA levels compared to controls. The effects of DIM on ER-α mRNA levels were detectable as low as with 1 μM.

### 3.5. Effects of AhR antagonists, α-naphthoflavone and luteolin, on the ER-α mRNA

AhR has been suggested as a possible regulator for ER-α expression [18,19]. To examine if AhR is involved with the suppressive effects of I3C/DIM on ER-α mRNA, MCF-7 cells were treated with AhR antagonists, α-NF and luteolin, with a concentration of 10 μM in the presence or absence of I3C or DIM (Fig. 3A and B). Both α-NF and luteolin that bind to AhR caused a significant decrease in ER-α mRNA levels. Adding I3C or DIM was unable to influence the response to this compound.

### 3.6. Effects of I3C and DIM on MCF-7 cell number homeostasis

An effect on cell number is often used to assess potential cancer preventive activity of a compound. Thus, we examined the changes in cell number in response to the I3C and DIM treatments, which may correlate with their modulating effects on ER-α expression. MCF-7 cells were treated with 0–100 μM of I3C for up to 72 h. Cell number was determined as described in Materials and methods. Significant inhibition ($P<.01$) of MCF-7 cell growth was observed after 72 h incubation with either 100 μM I3C (Fig. 4A) or 25 μM DIM (Fig. 4B).

## 4. Discussion

Cancer is associated with a host of environmental factors, including the daily exposure to the dietary component. A number of epidemiological and preclinical studies point to cruciferous vegetables as an important factor that can alter gene expression associated with breast tumor incidence and development [7,20,21]. Defining how these dietary constituents influence specific genetic pathways associated with breast cancer will help identify those who will benefit maximally from dietary intervention strategies.

Cellular behavior is determined by the expression of thousands of genes. Thus, monitoring multiple pathways involving transcription, oxidation, genomic instability, and cell cycle progression should provide additional clues about the effectiveness of dietary agents such as I3C [11,22]. High-throughput cDNA microarray analysis of cells treated with 100 μM I3C compared to controls suggests that cellular responses to I3C may involve the modulation of multiple pathways including DNA repair (e.g., BRAP), cell-cell interaction (e.g., GJA8), inflammation (e.g., NOS1) and hormonal regulation (e.g., ER-α). While some of these changes are consistent with data from Ashok et al. [11], other changes are not. Differences between these two sets of data likely reflect the different chips used, the cells examined and the duration of I3C exposure. Nevertheless, the suppressive effect of I3C on ER-α gene expression remains a consistent response.

A decrease in ER-α mRNA in response to I3C/DIM was confirmed with RT-PCR in our study. This effect of I3C/DIM on ER-α mRNA was found to be dependent upon the time of exposure and the concentration of these indoles. The reduction in ER-α mRNA appears to occur before the decrease in cell proliferation.

The mechanism by which I3C/DIM exerts their effects on ER-α mRNA remains unclear. Although the observed decrease in ER-α transcripts may reflect the I3C/DIM induced modification of the rates of synthesis, degradation, or both, it may also relate to secondary events in other transcription factors. I3C is recognized to have a relatively low affinity for ER-α, and thus the depression is likely not a direct effect. Previous studies have shown that the binding of AhR to its ligands including 2-, 3-, 7- and 8-tetrachlorodibenzo-*p*-dioxin causes the proteasome-dependent degradation of ER-α proteins in MCF-7 human breast cancer cells [18,19]. Since the binding of ligands translocates this receptor from the cytosol to the nucleus [23], the interaction between AhR and ER-α may also occur in the nucleus as well as in the cytosol. In the current study, when MCF-7 cells were treated with AhR ligands including α-NF (10 μM), luteolin (10 μM) and DIM (as low as 1 μM) for 72 h, there was a significant decrease in ER-α mRNA levels compared to controls. The antagonizing effects of α-NF and luteolin on the AhR were consistent with the previous observation [24,25]. The suppressive effect of 10 μM DIM on the ER-α mRNA is greater than that of 10 μM α-NF. The reason for this difference is not clear. Since DIM at concentrations less than 50 μM does not induce cytochrome P4501A1 gene expression [26], cytochrome P4501A1 is not likely to contribute to this difference.

In summary, our results clearly demonstrate that I3C and DIM, previously shown to inhibit estrogen-dependent breast cancer, cause a profound decrease in ER-α expression, possibly by interacting with the AhR. By doing so, estrogen-dependent signal transduction that results in breast cancer cell proliferation would be decreased, thus providing a biochemical and molecular basis for the known chemopreventive activity of these dietary agents.

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