Indole-3-carbinol inhibition of androgen receptor expression and downregulation of androgen responsiveness in human prostate cancer cells

Jocelyn C.Hsu, Joann Zhang, Anurupa Dev,
Aimee Wing, Leonard F.Bjeldanes¹ and
gary l.Firestone* 

Department of Molecular and Cell Biology and The Cancer Research Laboratory and ¹Department of Nutritional Sciences and Toxicology, The University of California at Berkeley, Berkeley, CA 94720, USA

*To whom correspondence and reprints should be addressed at: Department of Molecular and Cell Biology, 591 LSA, University of California at Berkeley, Berkeley, CA 94720-3200, USA.

Tel: +1 510 642 8339; Fax: +1 510 643 6791;
Email: glfire@berkeley.edu

Indole-3-carbinol (I3C), a naturally occurring compound found in vegetables of the Brassica genus, such as broccoli and cabbage, is a promising anticancer agent previously shown to induce a G1 cell-cycle arrest in the cells of human lymph node carcinoma of prostate (LNCaP) through regulation of specific G1-acting cell-cycle components. Since the androgen receptor (AR) mediates proliferation and differentiation in the prostate and is expressed in nearly all human prostate cancers, the effects of I3C on AR expression and function were examined in LNCaP cells. Immunoblot and quantitative RT–PCR assays revealed that I3C inhibited the expression of AR protein and mRNA levels within 12 h of indole treatment. I3C downregulated the reporter activity of LNCaP cells transiently transfected with an AR promoter-luciferase plasmid, demonstrating that a unique response to I3C is the inhibition of AR promoter activity. In contrast to I3C, the natural I3C dimerization product, 3,3'-diindolylmethane, which acts as an androgen antagonist, had no effect on AR expression. To determine the functional significance of the I3C-inhibited expression of AR, the AR-regulated prostate specific antigen (PSA) was utilized as a downstream indicator. I3C downregulated the expression of PSA transcripts and protein levels and inhibited PSA promoter activity, as well as that of a minimal androgen responsive element containing reporter plasmid. Expression of exogenous AR prevented the I3C disruption of androgen-induced PSA expression. Taken together, our results demonstrate that I3C represses AR expression and responsiveness in LNCaP cells as a part of its antiproliferative mechanism.

Introduction

Prostate cancer is the most prevalent cancer in men in USA, and the second most common cause of cancer-related death in males (1). Over the last 25 years, the number of men diagnosed each year with prostate cancer has increased by 30% (2). Treatment options for carcinoma of the prostate remain limited, and currently, the most common treatment—androgen ablation therapy—involves administering antiandrogens or castration to decrease the amount of circulating androgens (3,4). Given that prostate cancers initially develop as androgen dependent, removal of circulating androgens regresses the disease in 80% of patients (5). However, patients inevitably progress to an androgen-independent state of the disease ~18–24 months after administration of androgen ablation therapy, thereby becoming resistant to the treatment (3). It is important to note that the androgen-independent forms of prostate carcinomas typically express mutated androgen receptors (ARs) that respond not only to androgens, but also to other steroids and growth factors, even antiandrogens (6–9). Half of the patients who acquire resistance to androgen ablation therapy die within the first year following relapse, with the majority of the remainder succumbing to the disease within 2 years (1). The lack of therapeutics effective against all types of prostate cancers remains a critical problem in confronting the disease.

One promising anticancer agent, indole-3-carbinol (I3C), a naturally occurring compound found in vegetables of the Brassica genus, such as cabbage, broccoli and brussels sprouts, has been shown to reduce tumor occurrences in the colon, lung, skin, liver, cervix and mammary gland in mouse and rat models (10–14). Many studies have demonstrated that exposure to dietary I3C reduces the incidence and multiplicity of mammary tumors, although exhibiting negligible levels of toxicity (15–18). When ingested, I3C is converted into a variety of acid-catalyzed derivatives that probably account for the activity of dietary I3C (19,20). However, a number of studies indicate that I3C itself has potent antiproliferative activity. For example, ectopic application of I3C directly inhibits skin tumor formation in mouse models (12). Also, I3C induces a G1 cell-cycle arrest of estrogen responsive and unresponsive human breast cancer cell lines when administered directly to cultured cells (21,22).

We have previously demonstrated that I3C inhibited the growth of both AR-positive cell lines, such as lymph node carcinoma of the prostate (LNCaP) and MDA-PCa-2b, as well as AR-negative prostate cancer cell lines, such as DU-145, making I3C a candidate in the development of a therapeutic agent able to suppress the growth of both hormone-responsive and hormone-refractory tumors (23). This growth arrest was accompanied by regulation of expression or activity of specific G1 cell-cycle genes, such as cyclin-dependent kinases CDK2, CDK4, CDK6 and p21<sup>WAF1/CIP1</sup>. Furthermore, I3C downregulated the expression of prostate specific antigen (PSA), currently the most widely used diagnostic and prognostic marker for prostate cancer. PSA production and secretion often correlate with size and grade of prostatic tumors, making it a useful tool in determining the efficacy of novel therapeutics (24–27).

Abbreviations: AR, androgen receptor; ARE, androgen responsive element; CDK, cyclin-dependent kinase; DHT, dihydrotestosterone; DIM, 3,3'-diindolylmethane; GRE, glucocorticoid responsive element; I3C, indole-3-carbinol; LNCaP, lymph node carcinoma of the prostate; PSA, prostate specific antigen; TF, transcription factor.

Carcinogenesis vol.26 no.11 pp.1896–1904, 2005 
doi:10.1093/carcin/bgI155 
Advance Access publication June 15, 2005
An essential regulator in the normal development of the prostate and in the progression toward prostatic carcinoma is the AR. Its expression is present in nearly all prostate cancers, regardless of disease stage (28,29). Changes in the expression, activation, structure and function of the AR can lead to increased proliferation, and initiation or advancement of the disease (30). The AR, upon ligand activation by androgens, dimerizes and localizes to the nucleus where it can bind and activate the promoters of genes involved in proliferation and differentiation. Ligand-independent activation of the AR also contributes to disease progression (31–33). Although many drugs exist that disrupt the steroidal activation of the AR, either through ligand competition or diminishing the ligand supply in the bloodstream, there are very few compounds that target the expression of AR to interfere with the AR signaling pathway completely. In this study we demonstrate that I3C inhibits AR transcription and androgen responsiveness in human LNCaP cells.

Materials and methods

Materials

The human LNCaP cell line was purchased from American Type Culture Collection (Manassas, VA). RPMI Media 1640 with t-glutamine, HEPES buffer solution 1 M, and sodium pyruvate solution 100 mM were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum, calcium- and magnesium-free phosphate-buffered saline (PBS) and trypsin-EDTA were supplied by BioWhittaker (Walversey, MD). Dimethyl sulfoxide (DMSO), Me2SO, 99% high pressure liquid chromatography grade), I3C, 3,3-diindolylmethane (DIM) and tryptophol were purchased from Aldrich (Milwaukee, WI). Glucose solution of 40% concentration, chloramphenicol, chloramphenicol acetyltransferase (CAT) enzyme and dihydrotestosterone (DHT) were purchased from Sigma (St Louis, MO). An AR expressing adenovirus (AdCAT) was a kind gift from Phil Hart (US Environmental Protection Agency, NC) and a green fluorescent protein (GFP) expressing adenovirus (AdGFP) was a generous contribution of John Forte (University of California, Berkeley, CA). [3H]acetyl CoA was obtained from Perkin Elmer (Boston, MA). Antibodies for AR, tubulin and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-PSA antibody from DAKO (Carpinteria, CA). Oligonucleotide primers for PCR were purchased from Integrated Diagnostic Technologies (Corvial, IA). Luciferase reporter constructs pAR-luc and pARLuc-deltaKβ1-I were generous gifts of William Walker (Department of Cell Biology and Physiology, University of Pittsburgh, PA). PSA-lucis consists of the full-length PSA promoter cloned into pGL2-basic. pARE4-luc and pARE4-ehn-luc both consist of four AR binding sites cloned into pGL2-basic with the latter containing an additional AR binding site taken from the enhancer region of PSA-luc.

Methods of culture

LNCaP cells were cultured in RPMI 1640 medium with 2 mM of t-glutamine adjusted to contain 10% of fetal bovine serum, 4.5 g/l of glucose, 10 mM of HEPES, 1.0 mM of sodium pyruvate and 1.25 ml of 20 000 U/ml penicillin/streptomycin. Cells were propagated in a 37°C humidified chamber containing 5% CO2. Cell culture medium was changed every 48 h. 13C, DIM and tryptophol were dissolved in DMSO at concentrations 1000-fold higher than the final concentration in cell culture medium. Final concentrations of treatments in cell culture medium were 200 μM of I3C, 50 μM of DIM and 200 μM of tryptophol. These drug concentrations were determined by dose response treatments, and chosen for maximal response with minimal cell death. For infection with Ad5ShAR or AdGFP, cells were seeded 48 h prior to infection with 1 × 10^6 p.f.u. for 1 h with gentle rocking every 15 min and then for an additional 3 h. Infection media was then removed and cells were treated for 24 h as described below.

Flow cytometric analyses of DNA content

LNCaP cells were plated onto Corning six-well tissue culture dishes (Corning, NY) and were treated with the indicated concentrations of I3C, DIM and tryptophol. The medium was changed every 24 h. Cells were hypotonically lysed in 500 μl of DNA staining solution [0.5 mg/ml propidium iodide (PI), 0.1% sodium citrate, 0.05% Triton X-100 (Sigma)]. Lysates were filtered using 60 μm Nitex flow mesh (Sefar America, Kansas City, MO) to remove cell membrane and debris. PI-stained nuclei were detected using a PL-2 detector with a 575 nm band pass filter on a Beckman-Coulter (Fullerton, CA)

fluorescence-activated cell sorter analyzer 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/s. The percentages of cells within the G1, S and G2/M phases of the cell cycle were determined by analyzing the histogramic output with the multicycle computer program MPLUS, provided by Phoenix Flow Systems (San Diego, CA), in the Cancer Research Laboratory Microchemical Facility at the University of California at Berkeley.

Western blot analysis

After the indicated treatments, cells were harvested in PBS and lysed in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% nonidet P-40, 0.1% SDS and 50 mM Tris) containing protease and phosphatase inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 1 mM DTT, 0.1 mM sodium orthovanadate and 0.1 mM β-glycerophosphate). Total cellular proteins were quantified by the Bradford procedure and equal amounts of proteins were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 ml of 14.4 M 2-mercaptoethanol, 10% bromophenol blue and 3.13% stacking gel buffer) and fractionated by gel electrophoresis on 6, 10 or 12% polyacrylamide 0.1% SDS resolving gels. Rainbow marker (Amersham Pharmacia Biotech, Piscataway, NJ) was used as the molecular weight standard. Proteins were transferred to nitrocellulose membranes (Micron Separations, Westborough, MA) and blocked overnight at 4°C with 5% non-fat dry milk in 1× TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20). Blots were subsequently incubated with primary antibodies in 1× TBST for 1 h. Goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated secondary antibodies (Bio-Rad, Hercules, CA) were used at 1:5000 dilution in 1× TBST with 1% non-fat dry milk. Blots were treated with Western Blotting Western Blot Chemiluminescence Reagent (Perkin Elmer Life Sciences, Boston, MA) and the proteins were detected by autoradiography. Equal protein loading was ascertained by ponceau S staining of blotted membranes as well as western blotting with β-actin or tubulin antibodies.

Real-time quantitative RT-PCR analysis

After the indicated treatments, cells were rinsed with PBS and lysed with 1 ml of Tri Reagent (Sigma). Total RNA was prepared according to the manufacturer’s protocol and treated with DNase I, amplification grade (Invitrogen) to remove DNA contamination. An aliquot of 2 μg of total RNA was used to synthesize cDNA using Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI) with a random hexamer as a primer in a 20 μl reaction. Real-time quantitative PCR was performed using SYBR Green PCR Core Reagents and GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) using universal cycling conditions. Amplification reactions contained 5 ng cDNA template, components of SYBR Green PCR Core Reagents according to the manufacturer protocol and 300 nM of each primer in a final volume of 25 μl. The comparative threshold cycle (Ct) method was used to quantify data using β-actin as the normalizing gene. Human β-actin real-time PCR primers were purchased from Biosource (Camarillo, CA). Primers used for amplification of AR and PSA cDNA were carefully designed to span an intron to prevent amplification of genomic DNA and are as follows: AR fwd (5’-TGTTCAAATCTTCTACTTA-3’); AR rev (5’-CTGACTGTTCTGTTGCA-3’); PSA fwd (5’-GCCGACCCCAAGAAGTC-3’); PSA rev (5’-GCCGACCATCGATAAAG-3’).

Transfection and luciferase assay

For luciferase reporter assays, transfections were performed by mixing 0.8 μg of reporter plasmid with 6 μl of enhancer and 20 μl of Effectene reagent in Buffer EC according to the manufacturer’s protocol (Qiagen, Valencia, CA). Reactions were quenched after 5 h by removal of transfection medium and replaced by treatment medium. All transfections were done in triplicate. For luciferase assays, cells were harvested by rinsing with PBS and lysed in 500 μl of 1× Passive Lysis Buffer (Promega) for 15 min with gentle rocking. An aliquot of 2 μl of cell lysate and 8 μl of 2× Promega and subsequently loaded into a TD 20/20 Luminometer (Turner Biosystems, Sunnyvale, CA) after addition of 100 μl of Luciferase Assay Reagent II (Promega). Luminescence was measured in relative light units. The luciferase specific activity was expressed as an average of relative light units produced per microgram of protein present in corresponding cell lysates as measured by the Bradford Assay (Bio-Rad).

CAT assay

For CAT reporter assays, cells were transfected as described above. Cells were harvested in PBS and resuspended in 0.1 M Tris–HCl, pH 7.8. Samples were lysed by three freeze–thaw cycles and 150 μl of the cell lysate was incubated with 50 μl of 5 mM chloramphenicol, 25 μl of 1 M Tris–HCl, pH 7.8, 10 μl of [3H]acetyl CoA and 4 ml of Econofluor-2 in scintillation bottles. The reactions were incubated at 37°C for 1 h before counting with a scintillation counter. The relative CAT activity was measured in c.p.m. and normalized to the protein (μg) present in the cell lysates as measured by the Bradford assay.
Results

I3C decreases LNCaP cell proliferation and AR expression

To determine the antiproliferative effects of I3C compared with other indoles, human LNCaP cells were seeded at sub-confluence and treated with DMSO control, I3C, the I3C dimerization product DIM or with tryptophol, an inactive indole that differs in structure from I3C by only one methyl group. Cell proliferation was monitored by determining the cell-cycle phase distribution of PI stained nuclear DNA using flow cytometry. As shown in Figure 1A, cells treated for 24 and 48 h with I3C or DIM displayed a significantly decreased S-phase distribution of nuclear DNA compared with control cells treated with DMSO, whereas treatment with tryptophol had no effect on the number of S-phase cells. Consistent with our previous studies (23,34), treatment with either I3C or DIM arrested cell doubling, strongly repressed the incorporation of \(^{3}\)H]thymidine and induced an accumulation of cells in the G1 phase of the cell cycle (data not shown). AR levels were examined by western blots in cells treated with I3C, DIM, TRYP or with no indoles for 24 and 48 h. To control for gel lane loading, the level of AR was compared with that of tubulin protein. As shown in Figure 1B and C, I3C treatment strongly downregulated the production of AR protein. In contrast, DIM or TRYP had no effect on AR levels.

I3C downregulates AR transcript levels and inhibits AR promoter activity

To determine whether the downregulation of AR protein resulted from the loss of AR transcripts, the level of AR mRNA expressed in indole-treated and untreated cells was measured by quantitative RT–PCR of total RNA. As shown in Figure 2A, I3C downregulated the levels of AR mRNA as early as 6 h of treatment, with a maximal effect achieved by 12 h of indole treatment, which was maintained to the final 48 h time point. DIM or tryptophol had no effect on AR transcript levels (data not shown). Thus, the I3C-mediated loss of AR transcripts corresponded with and accounted for the downregulation of AR proteins.

The effects of I3C on AR promoter activity were utilized to determine whether the I3C downregulation of AR mRNA expression resulted from the disruption of AR gene transcription. LNCaP cells were transiently transfected with a vector containing a 1047 bp fragment of the AR promoter fused to a luciferase reporter gene. This promoter fragment contains the core promoter and is transcriptionally active in human prostate cancer cells (35) (diagrammed in Figure 2B). Luciferase activity was monitored in cells treated with or without indoles for 24 h. As also shown in Figure 2B, I3C treatment reduced AR promoter activity by 7-fold, which correlated with the loss in AR transcript expression. In contrast, treatment of LNCaP

Fig. 1. I3C causes growth arrest and downregulates AR protein expression. LNCaP cells were treated with DMSO vehicle control, 200 \(\mu\)M I3C, 50 \(\mu\)M DIM, and 200 \(\mu\)M tryptophol (TRYP) for 24 or 48 h. (A) Flow cytometry of LNCaP cells treated with various indoles. After treatment, cells were stained and analyzed for DNA content, separating cell population into different stages of the cell cycle. Percentages of cells accumulated in S-phase were graphed. (B) Western blot of AR. Total protein was isolated from treated LNCaP cells and immunoblotted with antibodies against AR or tubulin loading control. (C) Relative densitometry of western blot bands graphed as a ratio of target gene/tubulin control.
cells with DIM or TRYP failed to regulate AR promoter activity. These results demonstrate that a unique cellular response to I3C is the relatively rapid downregulation of AR gene transcription.

I3C treatment causes downregulation of downstream gene targets of AR

To determine the cellular consequences of the I3C downregulation of AR expression, production of the well characterized AR downstream target gene PSA was examined in indole-treated and untreated LNCaP cells. In these cells, the endogenous AR is mutated and can be activated by growth factors in the serum (9). Therefore, additional androgens do not need to be added to induce AR activity. Western blot analysis of cells treated with or without I3C for 24 and 48 h showed that I3C inhibited PSA protein production by 5-fold (Figure 3A). Tryptophol had no effect on PSA protein levels, demonstrating that this response was not a generalized indole effect. DIM inhibited the level of PSA protein despite the fact that DIM had no effect on AR protein expression. However, our previous studies revealed that DIM acts as a natural AR antagonist and thereby inhibits AR function (34). Since AR stimulates PSA transcription, quantitative RT–PCR was performed to measure the levels of PSA mRNA produced in indole-treated and untreated LNCaP cells (Figure 3C). Treatment with I3C or DIM rapidly decreased PSA transcript levels, with the near maximal response detected by 24 h of indole treatment. Thus, I3C and DIM each disrupt PSA expression.

Fig. 2. I3C downregulates AR transcript levels and promoter activity. (A) I3C inhibits levels of AR mRNA in LNCaP cells. Real-time quantitative RT–PCR for AR expression in I3C-treated LNCaP cells. Cells were treated with DMSO control or 200 μM I3C for indicated time points and total RNA were isolated and prepared as described. β-actin was used as a control and the graph represents the comparative C_{T} ratio of AR/β-actin. (B) Diagrammed is the AR-1047 promoter fragment fused to a luciferase expression vector. LNCaP cells were transfected with the AR promoter-luciferase vector and treated with DMSO control or 200 μM I3C, 50 μM DIM, 200 μM TRYP for 24 h. The cells were lysed, the extracts incubated with luciferase substrate reagent and the relative light units of the enzymatic reaction were read with a luminometer. The graph represents the fold-change of each indole treatment as compared with DMSO control.
but through distinct mechanisms involving either the inhibition of AR expression (via I3C) or inhibiting AR function as an antagonist (via DIM).

To confirm that I3C downregulates PSA gene transcription, LNCaP cells were transfected with a luciferase reporter gene containing a large PSA promoter fragment that included the core promoter with two AR DNA binding elements, AREI and AREII, as well as the enhancer region with a third AR DNA binding element, AREIII (Figure 4A). Transfected cells were treated with or without I3C for 24 h. As shown in Figure 4A, I3C induced a 4-fold inhibition of PSA promoter activity, which correlated with the time frame in which AR expression was significantly downregulated.

To test whether other downstream targets of AR would be affected similarly by I3C, LNCaP cells were transfected with a reporter plasmid containing a composite androgen responsive element (ARE) promoter fused to the luciferase gene. One of these vectors contained four AREs linked in tandem and the other vector contained the AREIII from the enhancer region of the PSA promoter linked upstream to the four tandem AREs (Figure 4B). As shown in the figure, I3C downregulated the reporter activity of both reporter plasmids. As a control to determine specificity of the I3C effect on AR-driven gene expression, a glucocorticoid responsive element (GRE) driven reporter construct (GRE–CAT) was employed (Figure 4C). I3C has no effect on the activity of the GRE-driven reporter construct despite its effect on AR regulated promoters. These results demonstrate that a functional consequence of the I3C-mediated downregulation of AR expression is the loss of promoter activity of downstream targets of AR.
Ectopic expression of AR prevented the I3C downregulation of AR production and disruption of androgen responsive PSA production

To determine whether the I3C inhibition of AR expression directly downregulates PSA expression, the full-length human AR was ectopically expressed in LNCaP cells from a constitutively active cytomegalovirus (CMV) promoter by infection using an adenovirus carrier. Unlike the endogenous AR in LNCaP cells, which is mutated and can be activated by growth factors in the serum, this exogenous wild-type AR requires an androgen ligand, such as DHT for activity. An adenovirus expressing GFP was used as a negative control along with cells that were uninfected with any adenovirus. The infected and uninfected cells were treated with combinations of I3C and DHT, and the levels of PSA, AR and tubulin control were examined by western blot analysis. In uninfected cells (adenovirus infection: none) and negative control infected cells (adenovirus infection: GFP) expressing only endogenous AR, treatment with I3C decreased AR and PSA protein levels in the presence of DHT (Figure 5, left and middle panels). These controls show that neither non-specific adenovirus infection nor DHT treatment itself had any effect on the ability of I3C to inhibit PSA expression. However, ectopic expression of AR abrogated the I3C downregulation of PSA protein levels (Figure 5, right panel). The high level of exogenous AR was not affected by I3C treatment since the ectopically introduced AR was driven by a constitutively active promoter. Under these conditions, I3C failed to downregulate PSA expression (Figure 5, right panel). Thus, activation of exogenous AR abrogated the I3C inhibition of AR target gene expression. These results demonstrate that the downregulation of active AR is a critical event for I3C regulation of PSA levels in LNCaP prostate cancer cells.

Discussion

The AR is a key regulator in the development and growth of prostate cancer and current therapeutic strategies utilize androgen antagonists that prevent AR activation, and/or disrupt endogenous androgen production (36–39). However,
in many instances these therapies ultimately fail as a result of AR activation by non-steroid physiological signals as well as the existence of mutant ARs in prostate cancers that can be activated by non-androgen steroids and certain growth factors (7,40–44). Our results demonstrate that I3C inhibits AR expression as a part of its growth arrest program in human LNCaP cells, a cell type that produces a mutant AR gene that is activated by androgens as well as by ER, EGF, and other hormones and growth factors (41–44). Thus, I3C is one of the very few compounds, such as resveratrol and the tea polyphenol EGCG (45,46) able to disrupt AR cellular activity by downregulating AR gene transcription. As such, I3C has the potential to be the basis for developing novel antiprostate cancer therapeutic strategies that utilize I3C compounds alone or in combinational therapies with other anticancer compounds. In this regard, we previously demonstrated that a combination of I3C and antiandrogen flutamide more effectively inhibit the growth of LNCaP cells than either compound alone (23).

In cultured human reproductive cancer cells, a portion of I3C is converted into its natural dimerization product DIM, suggesting that a subset of I3C responses is actually because of DIM activity (9). However, our results demonstrate that disruption of total cellular AR activity by I3C or DIM occurs by distinct mechanisms of action. As summarized in Figure 6, we propose that I3C inhibits AR promoter activity by disrupting the function or availability of specific transcription factors (TFs) that interact with this promoter. In contrast, DIM has no effect on AR expression or promoter activity, but rather this indole acts as an AR antagonist and blocks the activity of AR by competing with the ligand DHT (47). Thus, although either I3C or DIM can arrest the growth of LNCaP prostate cancer cells, I3C displays the distinct response of downregulating AR protein. Furthermore, this indole specificity demonstrates that the I3C effect on AR production is not an indirect consequence of the growth arrest. The resulting inhibition of functional AR through either regulation of expression or activity causes a loss of AR responsiveness and disrupts the high rate of cellular proliferation.

The I3C downregulation of AR expression and the DIM antagonism of AR activity in LNCaP cells should lead to a similar loss of AR downstream targets. PSA is a well-characterized AR-responsive gene with defined androgen regulated elements in its promoter (48). Consistent with their effects on AR expression and activity, treatment with either I3C or DIM strongly inhibited PSA expression and promoter activity. Since DIM directly binds to AR to inhibit its activity, whereas I3C downregulates AR expression, the regulation of the AR downstream target gene PSA should have different kinetics with each indole. Typically, changes in receptor activity are reflected more rapidly than altered expression. We observed that the initial effect of DIM on downregulating the level of PSA transcripts occurred after only 12 h of indole treatment, whereas the I3C effect was delayed at 18 h of treatment.

Although PSA has frequently been used as an indicator of AR responsiveness, there remained a possibility that I3C could have downregulated PSA expression by a mechanism independent of AR. To address this issue, we ectopically introduced AR driven by a constitutively active promoter and showed that the I3C inhibition of PSA expression was abrogated. This result confirms that the initial effect of I3C is to decrease AR expression and that this response is necessary and sufficient to regulate AR downstream targets.

An emerging concept from our work is that natural indoles with potent antiproliferative effects in human reproductive cancer cells act in part through the control of gene transcription. We have previously established that I3C inhibits expression of CDK6 in human breast cancer cells by disrupting the function of the Sp1 and ETS TFs at a composite element in the CDK6 promoter (22). Future key experiments are directed at determining the precise I3C responsive region in the AR promoter to establish which TF or sets of transcriptional regulators are targeted by the I3C signaling pathway. The elucidation of the I3C-regulated transcriptional signaling pathways would further the understanding of the mechanism of action of I3C and aid in the development of I3C and its related compounds as an antiprostate carcinoma therapeutic.

Acknowledgements

We thank Hanh H.Garcia and Kevin Hybiske for their helpful and critical comments during the course of this study. We are also very appreciative of Phil Hartig for his expertise in adenovirus infection and propagation. Grant Support: NIH Public Service Grant CA102360 from the National Cancer Institute. J.C.H. is a recipient of a predoctoral fellowship supported by NIH National Research Service Grant CA90941.

Conflict of Interest Statement: None declared.
References


Received March 24, 2005; revised May 20, 2005; accepted June 7, 2005