Introduction

It is clear that androgens are a major cause of prostate cancer (PCa). However, current evidence suggests that estrogen [17β-estradiol (E2) and its metabolites] is also a contributing factor [reviewed in references (1,2)]. It is well known that the incidence of PCa rises exponentially with increasing age. However, testosterone levels decline with increasing age, whereas E2 levels remain relatively unchanged (3-5), leading to an E2-dominant environment that contributes to prostate carcinogenesis (5,6). Additionally, African American men, who have the highest incidence of PCa in the United States, have serum E2 levels that are consistently higher than those in the general population but similar testosterone levels (7), whereas Japanese men have lower circulating E2 levels and a lower risk of PCa (8). In vitro, studies show that E2 increases proliferation in human PCa cells (9) and stimulates carcinoma in situ and adenocarcinoma in the prostates of rats and aging dogs (1,5,10,11). Furthermore, E2 metabolism has been shown to play a key role in prostate carcinogenesis [reviewed in (12)].

Lifestyle changes such as diet reduce or delay PCa. Genistein, a major soy isoflavone, and 3,3'-diindolylmethane (DIM), a major bioactive derivative of the dietary phytochemical indole-3-carbinol (I3C) from cruciferous vegetables, reduce the risk of PCa. Studies indicate that men whose diets are rich in soy (13,14) or cruciferous vegetables (15,16) have lower incidences of PCa. Laboratory studies, including translational studies, support the benefit of genistein, I3C, and DIM in providing a protective effect against PCa.

Genistein inhibits the growth of both androgen-dependent (17, 18) and androgen-independent human PCa cell lines (18,19) and can protect against chemically induced and spontaneously developing PCa in rodent models (20,21). In clinical studies, soy isoflavone supplementation prevents or decreases the rate of increase in prostate-specific antigen (PSA) levels in patients with PCa (22).
Likewise, I3C and DIM have potent antiproliferative effects in human PCa cells (23,24). In vivo, DIM inhibits tumor cell development, decreases cell proliferation, and induces apoptosis of prostate tumor cells (25). These findings are consistent with the observed protective effects of cruciferous vegetables in relation to PCa (16).

DIM and genistein have the ability to diminish the effects of E2 in hormone-related cancers. Previous studies in our laboratory demonstrated that both I3C and genistein decrease E2 signaling and that the effect is synergistic when these nutrients are used in combination (26). Furthermore, both nutrients favorably alter E2 metabolism away from the production of estrogenic and potentially carcinogenic estrogen metabolites (27,28). Taken together, these findings suggest that DIM and genistein may have the potential to reduce the negative effects of E2 on PCa.

Although some mechanisms by which these phytochemicals protect against PCa have been demonstrated, the ability of these nutrients to affect the impact of E2 on PCa has not been investigated, nor has the combined effect of these compounds on PCa been determined. This study investigated the effects of DIM and genistein individually and together on E2-induced proliferation, E2-induced gene expression, and estrogen metabolism in PCa cell lines.

Materials and Methods

Reagents
E2, 2-methoxyestrone, and genistein were purchased from Sigma. DIM was a gift from Dr. M. Zehligs (Bioreponse). Casodex (CSDX) was obtained from AstraZeneca.

Cell lines and cell culture
The human prostate adenocarcinoma cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection. All cells were maintained as monolayer cultures at 37°C in 5% CO2 and were grown in RPMI-1640 medium with glutamine (GIBCO-BRL) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories), 100 kU/L penicillin and streptomycin, and 200 mmol/L L-glutamine.

Cell proliferation assays
Cell proliferation was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation assay (Promega). Cells were seeded into 96-well plates at a density of 2000 cells per well in DMEM:F12(1:1) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories), 100 kU/L penicillin and streptomycin, and 200 mmol/L L-glutamine. The human prostate adenocarcinoma cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection. All cells were maintained as monolayer cultures at 37°C in 5% CO2 and were grown in RPMI-1640 medium with glutamine (GIBCO-BRL) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories), 100 kU/L penicillin and streptomycin, and 200 mmol/L L-glutamine.

Cell proliferation was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation assay (Promega). Cells were seeded into 96-well plates at a density of 2000 cells per well in DMEM:F12(1:1) phenol red-reduced media (GIBCO-BRL) supplemented with 2% charcoal-dextran-treated FBS (HyClone Laboratories). After 5 d, the cells were treated in separate experiments with the following: vehicle control (dimethylsulfoxide). For cytochrome P450 1A1 (CYP1A1), the forward primer was 5' CGGACCGTGATTGTTGAGA, the reverse primer was 5' AGCGAAGTGTATCGGTGAGA, and the Taqman probe was 5' CATTGCCGGCTGGAGGTCTTCTTCT. For catechol-o-methyltransferase (COMT), the forward primer was 5' CTGTTTGCCGTTGCTGGTAT, the reverse primer was 5' ATTGCCGCTGGTATTTGCTGC, and the Taqman probe was 5' TGCCCGGAGCTGTTGTTGGAG. Four replicates per condition were assayed and data averaged from 3 separate experiments are presented.

Androgen-response element–modulated transcription activity
To assess the effects of E2 on androgen-response element (ARE)–modulated transcription activity, analysis of luciferase reporter gene expression under control of ARE was performed. LNCaP cells (1 × 105 cells per well) were plated in 24-well plates in DMEM:F12(1:1) phenol red-reduced media supplemented with 2% charcoal-dextran-treated FBS and 24 h later they were transfected with the luciferase reporter vector (Translucent AR reporter vector, pAR-Luc). We used the FuGENE 6 Transfection Reagent from Roche to transfect pAR-Luc into LNCaP cells according to the manufacturer’s instructions. Experiments used 1 µg of pAR-luc for transfection and 0.02 µg of the renilla luciferase reporter plasmid pRL-TK (Promega) for normalization of transfection efficiency. Twenty-four h later the cells were treated with increasing concentrations of E2 (0, 1, and 100 nmol/L) for 48 h and then harvested for reporter gene assay with the dual luciferase reporter gene assay (Promega) according to the manufacturer’s instructions. Six replicates per condition were assayed and data averaged from 3 separate experiments are presented.

Prostate-specific antigen protein assay
LNCaP cells were seeded into 24-well plates at a density of 2.5 × 104 cells per well in DMEM:F12 (1:1) phenol red-reduced media (GIBCO-BRL) supplemented with 2% charcoal-dextran-treated FBS (Hyclone Laboratories) and allowed to attach for 24 h. Two d later, cells were treated with E2 and/or various concentrations of DIM and/or genistein for 5 d. After 5 d, the medium was collected from the treated cells and PSA protein concentrations were determined utilizing the Human PSA ELISA kit (Anogen). In the assay, the medium samples were incubated in 96 wells following the manufacturer’s instructions. The absorbance at 450 nm was measured with an Enzyme-linked microplate reader and analyzed using SoftMax Pro software. PSA values were then normalized to total protein utilizing the Micro BCA Protein Assay Reagent kit (Pierce). Six replicates per condition were assayed and data averaged from 3 separate experiments are presented.

RNA extraction and real-time quantitative RT-PCR
Total RNA was extracted from cell lines using the RNAeasy mini kit (Qiagen) with DNase treatment to remove any traces of genomic DNA. The relative expression of mRNA was determined using the Eurogentec RTqPCR mastermix (Eurogentec) and ABI PRISM 7700 Sequence Detection system (PE Biosystems). The PCR mix contained 1× master mix and 0.125 µL of Euroscript + RT and RNase inhibitor (RT-0.125 µL) and RNase inhibitor (0.05 µL/L). Taqman primers and probes were added at a final concentration of 500 nmol/L and 100 nmol/L, respectively. One hundred ng of total RNA was used per reaction in a 25-µL reaction volume. All samples were analyzed in duplicate. The thermal cycler conditions were 48°C for 30 min, 95°C for 10 min, and 45 cycles of 95°C for 0.15 min and 60°C for 1 min. Data were analyzed using Sequence Detection System software version 1.9.1. Results were obtained as threshold cycle (Ct) values. Ct is inversely proportional to the starting template copy number. β-Actin was used as reference gene and normalizer. Relative mRNA expression levels in samples treated with DIM and genistein were calculated compared with untreated control samples using the ∆∆Ct method (user bulletin no. 2, Applied Biosystems). Results are expressed as fold of the experimental control (dimethylsulfoxide). For cytochrome P450 1A1 (CYP1A1), the forward primer was 5’ AGCGAAGTGTATCGGTGAGA, the reverse primer was 5’ AATTCACACCCGGTTGAC, and the TaqMan probe was 5’ CATTGCCGGCTGGAGGTCTTCTTCT. For catechol-o-methyltransferase (COMT), the forward primer was 5’ CTGTTTGCCGTTGCTGGTAT, the reverse primer was 5’ ATGTTCCCGAGCTGTTGCTGC, and the TaqMan probe was 5’ TGCCCGGAGCCTGTTGTTGGAG. Four replicates per condition were assayed and data averaged from 3 separate experiments are presented.

GC/MS analysis of estrogen metabolites
Sample preparation for estrogen metabolite determinations. A 5-mL aliquot of media was diluted 1:1 with sodium acetate buffer (pH 4.6) and 20 µL of β-glucuronidase (110.2 u/mL) (Sigma). The solution was incubated at 40°C for 24 h to deconjugate the steroids. After the addition of deuterated estradiol (29), each sample was vortexed, extracted with chloroform, and evaporated completely. Each sample was derivatized by adding 10 µL of dry pyridine and 40 µL of bis(trimethylsilyl)trifluoroacetamide, vortexed, and allowed to react at room temperature overnight. One µL of each sample was injected into the GC-MS without further treatment.

GC-MS conditions. An Agilent Technologies 6890N gas chromatograph equipped with an Agilent 5973 mass selective detector, an Agilent 7863 injector, and an HP G1701CA MSD Chemstation was used for the analysis of E2 and selected metabolites [2-hydroxyestrone (2-OHE2) and 16a-hydroxyestrone (16a-OHE2)]. The method was modified from

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that of Adlercreutz et al. (30–33). Each of the above steroids was quantified using a 6-point calibration curve that ranged from 1 to 100 ng. Four replicates per condition were assayed and data averaged from 3 separate experiments are presented.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism and the data were analyzed by 1-way ANOVA with Tukey's post hoc comparisons. Data are expressed as means ± SD and differences were considered significant at $P < 0.05$.

**Results**

The effect of DIM and genistein on estrogen-induced proliferation in LNCaP cells. E2 dose-dependently increased the proliferation of LNCaP cells (Fig. 1A) in accordance with results by others (9), but E2 did not stimulate PC-3 cells ($P > 0.05$; data not shown). A direct cell count study was equivalent to the results of the MTS cell proliferation assay measurement (results not shown).

DIM decreased ($P < 0.0001$) E2-enhanced proliferation at all concentrations (5, 10, 15 μmol/L) (Fig. 1B). Treatment with the phytoestrogen genistein was more complicated. Genistein enhanced ($P = 0.02$) proliferation at 5 μmol/L but decreased the E2-enhanced proliferation at 10 μmol/L ($P < 0.0001$) and 15 μmol/L ($P < 0.0001$) (Fig. 1B). DIM (5 μmol/L) completely abolished the proliferative effect of genistein (5 μmol/L) (Fig. 1B).

**Estradiol stimulates the androgen receptor in LNCaP PCa cells.** E2 can bind the androgen receptor (AR) in LNCaP cells and some other PCa lines due to mutations in the AR (34,35). Our results indicated that the E2 stimulation of proliferation involved the AR for a number of reasons. First, both RNA coding for the AR and the AR protein were abundantly expressed in LNCaP cells but not PC-3 cells (data not shown).

Second, the antiandrogen compound CSDX, which binds to the AR, inhibited E2 stimulation of proliferation in LNCaP cells (Fig. 2A). Third, E2 increased expression of luciferase driven by the ARE and the endogenous AR (Fig. 2B). Finally, E2 increased the mRNA ($P < 0.05$; data not shown) and protein expression of PSA (Fig. 2C), which is an androgen-regulated gene. These results are consistent with E2 acting as an agonist for the AR.

**DIM and genistein decrease estrogen-induced PSA protein expression.** PSA is currently the most widely used tumor marker for PCa; increased levels of PSA correlate with an increased risk for developing PCa (36). Because E2 induced PSA gene (data not shown) and protein expression in LNCaP cells (Fig. 2C), we asked whether DIM and genistein inhibited this E2-induced expression. Both DIM and genistein significantly decreased estrogen-enhanced PSA protein expression at all concentrations (5, 10, and 15 μmol) (Fig. 3). DIM with or without genistein restored PSA protein expression to the baseline level (level without E2 enhancement) (Fig. 3).
**DIM and genistein alter E2 metabolism in LNCaP and PC-3 cells.** E2 metabolism can have favorable or detrimental effects on carcinogenesis, due to the different activities of the E2 metabolites that are generated (see Fig. 4). Moreover, E2 metabolism is independent of the steroid receptors, so both hormone-dependent as well as hormone-independent cancers could be affected. Because both DIM and genistein favorably alter E2 metabolism in other types of cancer (27,28), we hypothesized that they should have a positive effect on E2 metabolism in PCa cells. Utilizing quantitative real time RT-PCR, we found that both DIM and genistein increased the mRNA expression of CYP1A1 in LNCaP and PC-3 cells after 18 h of treatment (Table 1). The effect of the combination of the 2 phytochemicals was better than either alone. The combination of 15 μmol/L DIM and 15 μmol/L genistein significantly increased COMT mRNA expression in both cell lines, but neither alone increased expression of this enzyme (Table 1). However, at higher concentrations (25–50 μmol/L), both of these phytochemicals alone increased COMT mRNA expression in these cell lines (P < 0.05; data not shown). To determine whether changes in the mRNA expression of the E2 metabolizing enzymes correlated with changes in the E2 metabolites themselves, we measured the amount of the E2 metabolites 2-OHE2 and 16α-OHE1 that were secreted by LNCaP and PC-3 cells after treatment with DIM and/or genistein utilizing MS. The combination of DIM and genistein increased secretion of 2-OHE2 in LNCaP and PC-3 cells, a result that correlates with the increased CYP1A1 mRNA expression (Fig. 5A,B). Additionally, both DIM and genistein decreased estrogenic and carcinogenic 16α-OHE1 in PC-3 cells (P < 0.05; data not shown). This metabolite was not detected in LNCaP cells. Expression of CYP1B1 was increased by DIM (P < 0.05; results not shown), which could increase carcinogenic 4-hydroxyestrogens. However, no increase in this metabolite was observed in LNCaP and PC-3 cells (results not shown).

2-Hydroxylated estrogens are rapidly O-methylated by COMT to generate 2-methoxyestrone or 2-methoxyestradiol (proapoptotic as well as antiproliferative compounds). Whereas most of our analysis did not assay for the O-methylated compounds, a single assay did detect 2-methoxyestradiol in PC-3 cells after treatment with the nutrients (data not shown). In accordance with previous results (37), we found that 2-methoxyestrone increased apoptosis in both cell lines (P < 0.05; data not shown).

**Discussion**

Diets containing DIM and genistein reduce the risk of PCa. This study demonstrated that these phytochemicals, at least in combination, counteract the adverse effects of E2 by decreasing E2-induced proliferation and E2-induced PSA expression. Additionally, both phytochemicals drove E2 metabolism toward 2-hydroxylation and O-methylation in both the E2-sensitive LNCaP cells and the E2-insensitive PC-3 cells, a result that should decrease proliferation, decrease angiogenesis, and increase apoptosis.

Genistein, a phytoestrogen, has known E2 activity. Our results mimicked those in breast cancer cells in that low concentrations of genistein stimulated growth and higher concentrations inhibited growth (38). However, low concentrations of genistein (5 μmol/L) did cause a significant reduction in E2-induced PSA protein expression, indicating that low concentrations of genistein do in fact reduce the adverse effects of E2 on PCa. If genistein truly has a proliferative effect in regards to PCa, then DIM at low concentrations abrogates any proliferative effects caused by genistein when they are used in combination.

**TABLE 1** The effects of DIM and/or genistein on CYP1A1 and COMT mRNA in LNCaP and PC-3 cells after 18 h of treatment

<table>
<thead>
<tr>
<th>Phytochemical concentration</th>
<th>CYP1A1</th>
<th>COMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LNCaP cells</td>
<td>PC-3 cells</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>5 DIM</td>
<td>19.21 ± 0.59</td>
<td>18.71 ± 1.76</td>
</tr>
<tr>
<td>10 DIM</td>
<td>30.40 ± 3.34</td>
<td>40.63 ± 3.25</td>
</tr>
<tr>
<td>15 DIM</td>
<td>43.13 ± 12.95</td>
<td>38.16 ± 0.93</td>
</tr>
<tr>
<td>5 G</td>
<td>1.7 ± 0.27</td>
<td>1.13 ± 0.29</td>
</tr>
<tr>
<td>10 G</td>
<td>2.55 ± 0.04</td>
<td>1.47 ± 0.21</td>
</tr>
<tr>
<td>15 G</td>
<td>4.20 ± 0.11</td>
<td>3.33 ± 0.33</td>
</tr>
<tr>
<td>5 DIM, 5 G</td>
<td>31.73 ± 8.46</td>
<td>20.04 ± 3.98</td>
</tr>
<tr>
<td>10 DIM, 10 G</td>
<td>66.65 ± 5.86</td>
<td>31.60 ± 6.80</td>
</tr>
<tr>
<td>15 DIM, 15 G</td>
<td>108.81 ± 19.62</td>
<td>26.74 ± 0.61</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 3. Means without a common letter differ, P < 0.05 (Tukey’s test).
2 G, genistein.
The precursor to DIM, and genistein (synergistic together) are applicable to this study, our previous studies indicate that I3C, carcinogenesis [reviewed in (2,41,42,51,52)]. Although not others indicate that both ER
psa expression. Although genistein does not bind to the AR, it to bind to the AR (49), leading to decreased proliferation and DIM, this decreased stimulatory effect may be due to its ability increasing estrogenization of older males. In these studies, DIM postulate that a selection for E2-sensitive AR occurs due to the stimulation being related to the AR. More PCa lines are being stimulated by E2 (34,35). Our studies were consistent with E2 were not expressed in the LNCaP cells, the cells did abundantly and genistein, appeared to be related to the AR. This was evident in PC-3 cells. Values are means ± SD, n = 3. Means without a common letter differ, P < 0.05.

The sex-hormone receptor status of the prostate, with respect to PCa, is complicated. In the prostate, AR and estrogen receptors (ER) are expressed and all seem to play a key role in prostate carcinogenesis (39–42). The E2 stimulation of proliferation in LNCaP cells and, conversely, its inhibition by DIM and genistein, appeared to be related to the AR. This was evident by the fact that the LNCaP cells used in this study did not express either ERα or ERβ, albeit we did detect ERβ (but not ERα) in LNCaP cells from another laboratory (data not shown). It is not uncommon for cells to lose expression of their ER (43–45); loss of receptor expression is one of the most important steps in acquiring hormone resistance (46,47). Although, the ER were not expressed in the LNCaP cells, the cells did abundantly express the AR, which contains mutations allowing it to be stimulated by E2 (34,35). Our studies were consistent with E2 stimulation being related to the AR. More PCa lines are being identified with mutated AR that respond to E2 (35,48), One can postulate that a selection for E2-sensitive AR occurs due to the increasing estrogenization of older males. In these studies, DIM and genistein reduced the stimulatory effects of E2. In the case of DIM, this decreased stimulatory effect may be due to its ability to bind to the AR (49), leading to decreased proliferation and PSA expression. Although genistein does not bind to the AR, it may accomplish these same results via its ability to cause downregulation of the AR (50). In addition to the AR, studies by others indicate that both ERα and ERβ are involved in prostate carcinogenesis [reviewed in (2,41,42,51,52)]. Although not applicable to this study, our previous studies indicate that I3C, the precursor to DIM, and genistein (synergistic together) are negative regulators of ERα (26), suggesting that both phytochemicals have the ability to provide a protective effect against the negative effects of E2 that are related to the ER. Putting this information together, DIM and genistein should downregulate E2 stimulation by both the AR and ER.

A clear link between E2 metabolism and prostate carcinogenesis exists (12,53,54). E2 metabolism is independent of the ER and AR, and modulation of E2 metabolism by DIM and genistein could have a positive affect on any prostate cell regardless of its hormone status, resulting in a reduction of carcinogenic estrogen metabolites and generation of metabolites that are antiproliferative, proapoptotic, and antiangiogenic. Both DIM and genistein increase the expression of CYP1A1 and COMT in other systems and increase 2-hydroxylation in vivo (27,28,55). In this study, we showed that DIM and genistein increased expression of these enzymes in PCa cells. We also showed that both phytochemicals increase 2-OHE2, which has weak estrogenic activity (56) and is rapidly O-methylated, while simultaneously decreasing 16α-OHE1, which has prolonged estrogenic activity (57,58). The implications of these favorable alterations in estrogen metabolism in PCa has been documented in vivo. For example, a case control study of urinary estrogen metabolites and PCa indicated that elevated 2-OHE2 urinary levels are associated with a reduced risk of developing PCa, whereas elevated 16α-OHE1 urinary levels are associated with an increased risk of PCa (53).

This study further supports the notion that DIM and genistein will help efforts against PCa, showing that both these phytochemicals diminish adverse effects of E2. Effective concentrations are difficult to compare in vitro vs. in vivo and further studies are needed to determine which in vitro concentrations of these phytochemicals would be most beneficial. Importantly, our combination studies indicate that much lower concentrations of the phytochemicals can be used to achieve a favorable outcome. DIM is known to concentrate in tissues after administration (59) and recent clinical studies reveal that significant elevations of intraprostatic genistein can be achieved with short-term dietary phytoestrogen supplementation (60). Together, the implication is that it should be possible to achieve concentrations of DIM and genistein, especially in combination, that would be beneficial in clinical chemopreventative strategies targeting PCa.

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Literature Cited


