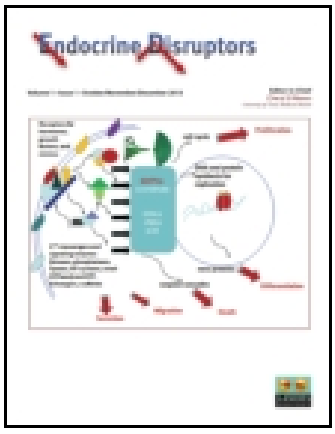

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Rapid, nongenomic signaling effects of several xenoestrogens involved in early- vs. late-stage prostate cancer cell proliferation

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Keywords: BPA, coumestrol, diet, genistein, membrane estrogen receptor, resveratrol

Xenoestrogens (XEs) are exogenous mimics capable of binding to estrogen receptors (ERs), competing with/disrupting the actions of physiological estrogens, and promoting tumor growth in the prostate and other endocrine tissues. Humans are exposed to numerous XEs including environmental contaminants such as plastics monomer bisphenol A (BPA), and dietary phytoestrogens such as coumestrol and genistein from soy, and resveratrol, highest in red grapes. There is growing interest in the ability of phytoestrogens to prevent or treat tumors. We previously reported that multiple cellular mechanisms influence the number of prostate cancer cells after estradiol or diethylstilbestrol treatment. We now examine the effect of these XEs on signaling mechanisms that alter the number of LAPC-4 (androgen-dependent) and PC-3 (androgen-independent) cells at environment- and diet-relevant concentrations. Coumestrol and genistein both increased the number of LAPC-4 and PC-3 cells dramatically. Rapid alterations of phospho- and total-cyclin D1 levels most closely correlated with the XE-induced changes in cell numbers. Sustained activation (phosphorylation) of the extracellular signal-regulated kinases 1 and 2 as a prelude to generation of reactive oxygen species also partially contributed to the XE's effects on cell numbers. Early-stage cells expressed higher levels of all 3 ERs (including those in membranes) than did late-stage cells; ER subtypes were variably involved in the signaling responses. Taken together, these results show that each XE can elicit its own signature constellation of signaling responses, highlighting the importance of managing exposures to both environmental and dietary XEs for existing prostate tumors. These mechanisms may offer new cellular targets for therapy.

Introduction

Prostate cancers are well-known for their initial androgen responsiveness, which diminishes with the progression of disease stage. While the corresponding decrease in androgen receptor (AR) levels that accompanies this decline in responses has been well documented,[1] little is known about the relationship of tumor progression with the estrogen receptor (ER) types that might be involved, such as those that are thought to mediate the therapeutic effects of the pharmaceutical estrogen diethylstilbestrol (DES). There are many types of xenoestrogens (XEs) – exogenous estrogen-like compounds that bind to ER ligand binding pockets.[2-4] In normal or cancer cells, they imitate, compete with, or disrupt the actions of physiological estrogens.[5,6] Some XEs are known to promote tumor development in many tissues by stimulating inappropriate endocrine responses via ERs, promoting angiogenesis, increasing DNA adducts, or altering the epigenome.[7-12] Actions of XEs via ERs have also been shown to cause the proliferation of established endocrine tumors or tumor cell lines of many types, including those from brain, breast, kidney, lung, pancreas, prostate, and testis.[13-20]

Alternatively, some XEs, especially dietary compounds, have been credited with preventing tumors in some of these tissues,[21–24] highlighting a broad range of XE response profiles. Genistein is a phytoestrogen found in soy products, fava beans, and some coffee bean preparations[25] that can cause cell cycle arrest and growth inhibition at concentrations within the ranges achieved by the diets of some cultures (10^{-8} M to 10^{-6} M), via the down regulation of cyclin B.[26,27] Coumestrol (found in red clover, alfalfa sprouts, and also some soy products) can kill breast and colon cancer cells by producing reactive oxygen species (ROS).[28] Resveratrol, found in grapes, can decrease cell

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numbers by increasing intracellular calcium levels, disrupting G1/S progression, and stimulating apoptosis.[29-33]

XEs also include environmental contaminants from the manufacture, use, or leachates of consumer products (e.g. plastics, chlorinated pesticides, alkylphenol surfactants). Bisphenol A (BPA) is a component of many plastic products (such as water bottles, food containers, receipt paper, and the inner coatings of food cans [34]) and leaches from these products more readily with heat or acidity. As a result, BPA is a common environmental and human/animal contaminant [35] that has been shown to alter cell proliferation,[36] cell signaling through the activation of mitogen-activated protein kinases (MAPKs)[36-38] and intracellular calcium levels,[39,40] prostate cancer cell migration,[41] and increase susceptibility to certain diseases.[7,42]

Epidemiological studies generally support an association between diets high in phytoestrogens and low cancer incidence.[43–45] African-Americans have a higher incidence of prostate cancer,[46] and dietary differences are being investigated as a possible factor in tumor development and progression within that population. East Asians consume high amounts of phytoestrogens, and their incidence of many types of cancer, including prostate cancer, is much less.[45] Asian diets contain high levels of soy ingredients, with the best-known active estrogenic components being daidzein, genistein, and coumestrol.[47] Genetics may also play a role in the sensitivity of various cancer-relevant mechanisms to estrogens, including the ability to metabolize dietary phytoestrogens to more active compounds,[48] though this can also be due to the type of gut microbiome present.[49]

Until recently, most studies on XEs were focused on the gene expression (genomic) consequences of exposures.[50-52] Even at high concentrations, XEs generally elicited only low levels of transcriptional responses, so these compounds were thus labeled as weak estrogens.[50,53] However, XEs can also initiate non-genomic responses, so classifying them as weak without taking these more rapid cellular responses into consideration may be misleading.[15,20,54-58] Some endogenous estrogen metabolites such as estriol were formerly labeled as weak because of their limited ability to activate specific transcription, but have recently been found to have profound effects on disease expression.[59,60] Estriol, like XEs can have quite potent effects on nongenomic responses.[61] Because of this belief that XEs were weak, many past studies did not evaluate estrogens at environmentally relevant low doses (reviewed in[62]). Dose responses to estrogens are typically non-monotonic and therefore must be assessed over a wide and detailed range of concentrations (reaching down to the femtomolar to nanomolar range) to predict their ability to act, especially at relevant environmental and dietary levels.[9,20,61,63]

We have previously shown that XEs can rapidly activate cellular signaling pathways in tumor cells of other tissues (pituitary, breast, adrenal), and when in combination with them can modify the actions of physiological estrogens.[37,38,55,64-66] MAPKs can be rapidly activated or deactivated by XEs, leading to alterations in such functional end points as proliferation, apoptosis, and prolactin release.[20,67] Moreover, these MAPK phosphorylations [such as those for the extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinase] often occur at low physiological estrogen and XE concentrations.[20,37,68]

We also recently demonstrated that estradiol (E_2) and diethylstilbestrol (DES) can rapidly stimulate or deactivate ERKs in LAPC-4 and PC-3 prostate cancer cells,[69] and when sustained, cause ROS generation, contributing to a decrease in viable cells.[69,70] In addition, estrogen-induced rapid phosphorylation of cyclin D1 led to its subsequent prompt degradation, which in turn was correlated to the ability of E_2 and DES to inhibit growth of these cells.[69] We will now investigate if some XEs also alter the viability of prostate cancer tumor cells via these mechanisms. Elucidating how these XEs function in early- vs. late-stage prostate tumor cells could lead to selective advice for patients about diet and exposure to environmental estrogens.

Materials and Methods

Cell lines and hormones

We chose cell lines representing the 2 main types of prostate cancers – androgen-dependent vs. androgen-independent. LAPC-4 androgen-dependent prostate cancer cells (passages 45–50)[71] were maintained to sub-confluence in phenol red-free Iscove's Modified Dulbecco's Medium (IMDM; MediaTech, Manassas, VA) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 4 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), and 10^{-9} M dihydrotestosterone (Sigma-Aldrich). PC-3 androgen-independent prostate cancer cells (passages 18–23)[72] were maintained by growth in phenol red-free RPMI 1640 (Sigma-Aldrich) with 10% FBS and 2 mM L-glutamine. Both cell lines were propagated at 37°C in 5% CO_2. BPA, coumestrol, genistein, and resveratrol (all from Sigma-Aldrich) were dissolved in ethanol to a stock concentration of 10 mM before serial dilution into IMDM or RPMI 1640 at concentrations ranging from 10^{-14}M to 10^{-6}M (and a final EtOH concentration of 0.0001%).

MTT cell viability assay

Cells were plated at 5,000 cells/well in poly-D-lysine-coated (BD Biosciences, Bedford, MA) 96-well assay plates, (Corning, Tewksbury, MA), and then allowed to attach overnight. The next day, XE treatments were added in 100 μL of medium with 1% 4x charcoal-stripped FBS. Extensive charcoal stripping of serum was done to remove and thus minimize the effect of any steroid hormones already present; these conditions were previously optimized to demonstrate effects of steroids and mimics on cell proliferation for these cell lines. After three days, treatments were removed and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was added for 1 h. Cells were then lysed and the signal read at 590 nm in a Wallac 1420 plate reader (Perkin Elmer, Waltham, MA).

Plate immunoassays

Phosphorylated proteins were recognized by antibodies (Abs) specific for these post-translationally modified epitopes: pERK1/2 (Thr202/Tyr204) and phospho-cyclin D1 (Thr286) (both

from Cell Signaling, Danvers, MA). Changes in total cyclin D1 levels were measured by using an Ab to cyclin D1 recognizing both modified and unmodified protein cyclin D1 (Cell Signaling Cat. No. 2922). ER Abs used included ERα (MC-20, Santa Cruz Biotechnology, Dallas, TX); ERβ (clone 9.88, Sigma-Aldrich); and GPR30 (Cat. No. NLS4271, Novus Biologicals, Littleton, CO). Membrane and total ER levels were measured by controlling for permeabilization of the cell membrane. A plate immunoassay developed in our lab[73] and used in many of our past studies was recently adapted[74] for use with the BIOMEK FXP workstation (Beckman Coulter, Brea, CA) to automate the majority of the plate assay's liquid handling, decreasing experimental variability and increasing experimental output.

Prostate cancer cells were plated at 10,000 cells/well in 96-well assay plates, allowed to attach overnight, and given 100 μL of medium with 1% 4x charcoal-stripped FBS for 48 h. Cells were then treated with XEs for up to 60 min on the workstation, followed by fixation (2% paraformaldehyde, 1% gluteraldehyde) ± permeabilization (0.15 M sucrose, 0.5% Nonidet P-40; to access internal vs. extracellular epitopes). The primary Ab to the phosphorylated epitopes was then added and incubated with the cells overnight. The next day, biotinylated anti-mouse/anti-rabbit IgG secondary Ab (Vector Labs, Burlingame, CA) was added for 1 h. Next, cells were incubated for 1 h with avidin-biotinylated conjugated alkaline phosphatase (ABC-AP, Vector Labs), then for 30 min with para-nitrophenylphosphate substrate (Thermo Scientific, Rockford, IL), allowing the yellow color of the para-nitrophenyl product to accumulate. Plates were read at 405 nm in a Wallac 1420 plate reader. Readings were then normalized to cell number, estimated by the crystal violet dye (Sigma Aldrich) assay as described previously.[73]

Subtype-selective ER antagonist assays

To further determine ER subtype involvement in altering ROS formation or cyclin D1 phosphorylation, the following ER antagonists were used at their receptor-selective concentrations: for ERα, 10^{-7}M MPP; for ERβ, 10^{-6}M PHTPP; and for GPR30, 10^{-6}M G15 (all from Tocris Bioscience, Minneapolis, MN). All were dissolved in ethanol to a stock concentration of 10 mM, then serially diluted into culture medium. Final ethanol concentrations were 0.0001%, which was used as vehicle control for all studies. Cells were incubated with antagonists for 30 min before XE treatments.

ROS assays

Cells were plated at 10,000 cells/well in a 96-well assay plate, then allowed to attach overnight. Cells were then treated with 100 μL of medium containing 1% 4x charcoal-stripped FBS for 48 h. 2',7'-Dichlorodihydrofluorescein diacetate (DCDHF, Enzo Life Sciences, Farmingdale, NY; 15 μM) was loaded into cells for 1 h, and XE treatments were then administered for 15 min. Hydrogen peroxide (Fisher Scientific, Pittsburg, PA) and ethanol (0.0001%) were used as positive and negative controls, respectively. E_2 (1 nM) was a positive control for previously determined estrogenic responses.[69] Dichlorofluorescein production, formed as a result of ROS/DCDHF interaction, was measured at an excitation of 485 nm, and an emission of 538 nm in a SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices).

Statistics

All experiments were conducted a minimum of 3 times. One-way analysis of variance was conducted for all experiments except ER quantification, which was analyzed using a Student's *t*-test. A Holm-Sidak post hoc test was used to measure the significance of each treatment versus the vehicle control. Significance was set at $P < 0.05$, unless otherwise stated.

Results and Discussion

XE effects on the number of viable cells

XEs at environment- or diet-relevant concentrations caused some increases in the numbers of LAPC-4 and PC-3 prostate cancer cells, observed here after 3 d of exposure in media containing 1% charcoal-stripped serum (**Figs. 1A & B**). Coumestrol increased viable cell numbers at all but the lowest concentration assessed (10^{-14}M) in both cell lines (by >200% in LAPC-4 cells, >400% in PC-3 cells). There was a strikingly different response to genistein between cell lines representing different tumor stages; genistein did not affect LAPC-4 cells, while it strongly stimulated

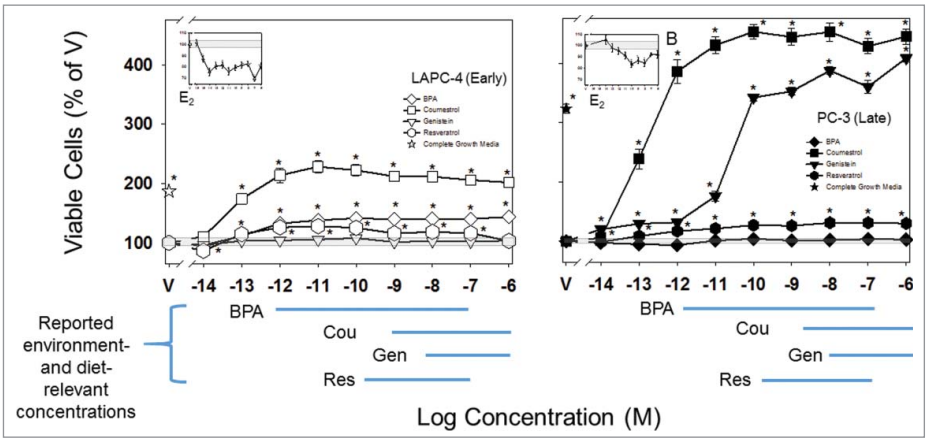

Figure 1. Cell number after 3 d of XE treatment. LAPC-4 and PC-3 prostate cancer cells were treated with XEs and viable cells were measured by the MTT assay. In all figures throughout the manuscript white symbols denote LAPC-4 cells and black symbols PC-3 cells. *denotes significance from vehicle (V) controls at $P < 0.05$, and shaded horizontal bars represent the response to V ± SEM. In this and other graphs, where error bars are not visible, they were within the size of the symbol. Dietary or environmentally relevant concentration ranges are shown by the solid horizontal bars below the graphs for each XE. The insets show cell numbers after 3 d of E_2 treatment, for comparison (and see [69]).

the growth of PC-3 cells (by ~4-fold at concentrations from 0.1 nM to 1 μM, all levels achievable by some diets). BPA caused a small stimulatory effect in LAPC-4 cells, at concentrations $>10^{-12}$M (maximal increases of ~40–50%), but had no effect on PC-3 cells. Resveratrol showed minimal, but significant stimulation compared to vehicle in both cell lines at most concentrations tested. For comparison to other estrogens assessed in our other studies[69] (see insets), even at physiological (10^{-10}–10^{-8}M) concentrations E_2 instead caused a significant decrease in cell viability for both cell lines by ~20–30%. The actions of these dietary estrogens in causing prostate cancer cell growth are perhaps unexpected, given the epidemiological evidence that cultures with diets rich in genistein and coumestrol show decreased levels of prostate cancer.[75,76] However, some other studies have also shown a prostate cell proliferation effect by these phytoestrogens.[77–79]

Subcellular location and levels of ERs

We next asked which types of ERs were present in LAPC-4 and PC-3 cell lines that could mediate these changes in viable cell number. We had previously noted a variable dependence of rapid responses on all 3 ER subtypes (determined by using selective antagonists) in our studies on E_2 vs. DES treatment of these cells.[69] Plasma membrane versions of estrogen receptors (mERs) are thought to mediate rapid signaling involved in cell number changes in other cell types [reviewed in[64,80,81]], so we examined the subcellular location of these receptors here. Using our plate immunoassay ± cell permeabilization with detergent (**Fig. 2**), we observed that late-stage tumor cells (panel B) had much lower expression of ERs than did early-stage cells (panel A; note the ~fold6- vertical scale difference between panels A and B), a frequent finding among steroid receptors in endocrine cancers of multiple types,[82-84] although this evaluation for mERs in prostate tumor cells is novel. We also saw that membrane receptor populations were much lower than total (and thus intracellular) receptor forms in early-stage cells; the levels of mERs α and β were about 20% and 24% of their total receptor populations, respectively, as we have seen previously for the proportion of membrane versions of these receptors in other tumor cell types.[85-87] Although much lower, we detected significant levels of all 3 ER types in PC-3 cells. In these late-stage tumor cells the membrane receptor population was a much larger percentage of the total receptor numbers, perhaps in keeping with their more undifferentiated state, as we have seen with membrane glucocorticoid receptors in human lymphoma cells compared to normal circulating lymphocytes.[88] ERβ predominated in LAPC-4 cells (and to a lesser extent in PC-3 cells), as expected based on the literature regarding the dominance of this receptor type in normal prostate tissues and the early-stage tumors that arise from them.[89,90] However, there were also significant levels of ERα and GPR30, suggesting that they might also play a role in mediating estrogenic mechanisms. Interestingly, we found that the sizable amount of GPR30 was largely intracellular in LAPC-4 cells. GPR30 has been identified in other prostate cancer studies, but the subcellular location was not elucidated.[91-93] The subcellular location of GPR30 in other tissues and their cancers has been a point of contention; different groups have demonstrated this receptor form as either primarily in the plasma membrane or in the endoplasmic reticulum.[94,95]

Phospho-ERK

Our next goal was to identify pathways and mechanisms responsible for any changes in numbers of viable cells, and ERK phosphorylation is one mechanism that has traditionally been associated with cell proliferation.[70] We selected an effective and environment- or diet-relevant concentration for each XE studied (10^{-9}M BPA, 10^{-7}M coumestrol, 10^{-7}M genistein, and 10^{-8}M resveratrol; see **Fig. 1** for relevant ranges), and measured their ability to elicit ERK phosphorylation in both cell lines over 60 min (**Figs. 3A and B**). We observed activation for all compounds except genistein, but found that a sustained (60 min) pERK response did not predict a XE's positive influence on cell number, as has been a long-held association.[96,97] The most striking result was the difference between cell line-specific responses after resveratrol treatment, which caused a strong ERK deactivation (40%) in LAPC-4 cells, while it had slightly increased the number of viable cells (**Fig. 1**). In PC-3 cells resveratrol rapidly activated and sustained pERK (at 60 min), and caused modest cell proliferation. BPA and coumestrol rapidly stimulated ERK phosphorylation in both cell lines (**Figs. 3A and B**), all with sustained levels at 60 min, but had no proliferation effects in late-stage cells. Genistein rapidly though modestly deactivated ERK in both cell lines, but substantially increased viable PC-3 cell numbers. Only the activation of ERK by coumestrol in both cell types correlated with its ability to cause these cells to proliferate. Therefore, these XEs

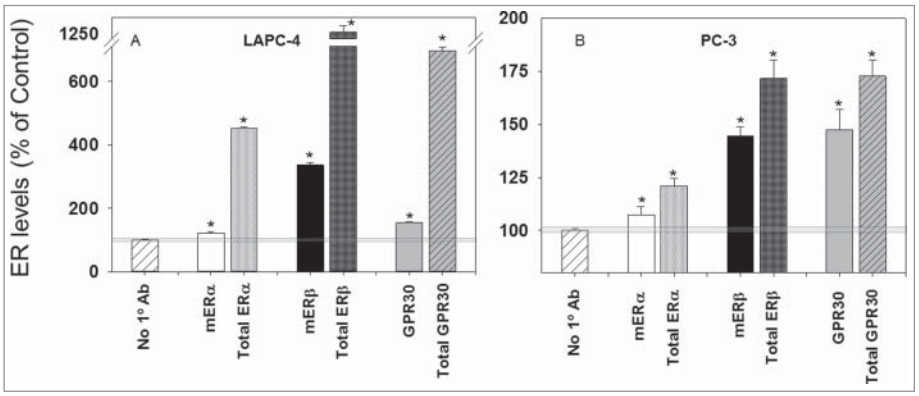

Figure 2. ER subtype (α, β, and GPR30) levels (total vs. membrane) in LAPC-4 and PC-3 prostate cancer cells. The negative control samples used no primary antibody (Ab) for any of the ER subtypes, as indicated by the first bar and the shaded bar extending horizontally across the graph (average ±SEM). *denotes significance from controls at $P < 0.05$.

elicited unique patterns of ERK activation/deactivation, which could contribute to cell survival, cell death, or proliferation,[70,96,98] but clearly more mechanisms needed to be considered.

ROS generation

Others have recently associated sustained ERK activations with ROS generation leading to cell killing,[70] and we recently extended this to the ability of a physiological estrogen (E_2) to induce cell death in early-stage prostate cancer cells.[69] Therefore, we examined if XEs (at optimal concentrations for such ERK responses) could be linked to any ROS elevations (measured at the peak time of 15 min, time course not shown). The positive controls for ROS generation, including both H_2O_2 and E_2, caused robust ROS generation (**Figs. 4A and B**), and as we saw previously, cell death.[69] Most XE treatments generated significant ROS levels regardless of whether they had caused sustained ERK activation (**Fig. 3**), though BPA and genistein did so only in one cell line each. These ROS increases were all modest compared to those caused by E_2. Therefore, ROS elevation due to sustained ERK activation was not considered to be a primary mechanistic determinant of viable cell number in these studies. Perhaps higher levels of ROS need to be generated to kill this cell type.

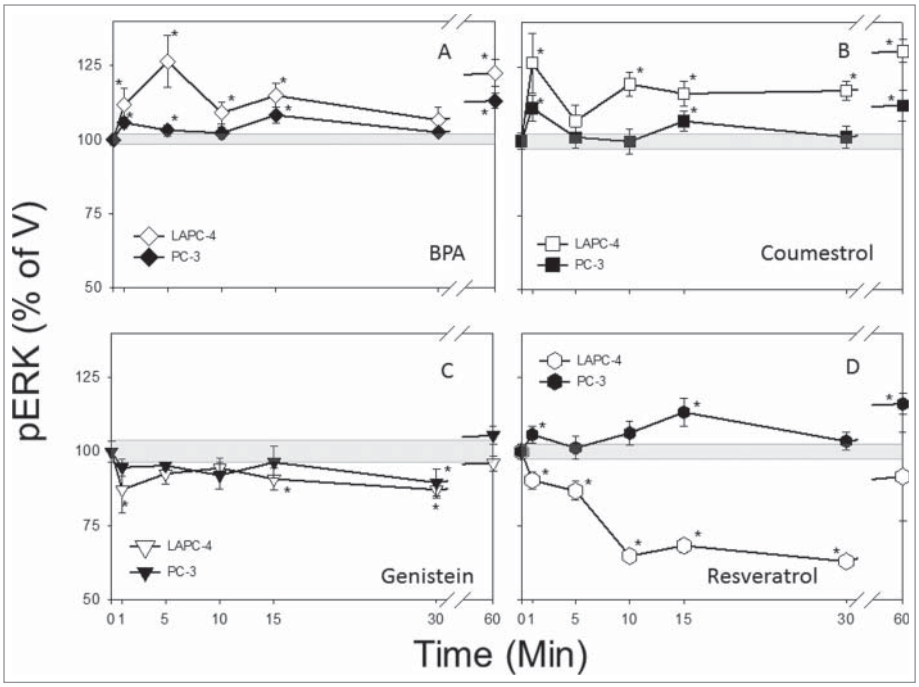

Figure 3. Phospho-ERK (pERK) levels in LAPC-4 and PC-3 cells after XE treatments. LAPC-4 and PC-3 cells were treated with 10^{-9}M BPA, 10^{-7}M coumestrol, 10^{-7}M genistein, and 10^{-8}M resveratrol. pERK was measured up to 60 min via the plate immunoassay. *denotes significance from vehicle (shown at time 0) controls at $P < 0.05$, and horizontal shaded bars represent the response to vehicle ± SEM.

Therefore, these MAPK, and potentially linked ROS responses, did not individually predict cell proliferation vs. cell killing effects. Possibly a more traditional route of ROS generation not involving ERKs was involved in these cells. Estrogens have been shown to damage DNA,[99] which can also cause ROS generation.[100,101] The ability of these XEs to induce ROS was different from that of E_2,[69] further highlighting the imperfect mimicry of physiological estrogens by XEs. Overall, the pERK and ROS responses to XE treatments in both cell lines do not appear to be lone driving mechanisms that elicit changes in cell numbers. Therefore, we have to consider the combined contribution of these responses to an overall balance of competing mechanisms (see below).

We observed previously that E_2 required different ER subtypes to elicit ROS responses in LAPC-4 vs. PC-3 cells.[69] Here XEs also demonstrated unique ER-use signatures for this response (**Fig. 4**). In LAPC-4 (early-stage) cells, BPA caused these modest ROS increases independent of any known ERs, and genistein did not raise ROS levels (**Fig. 4A**). The increases due to coumestrol required ERα and GPR30, while resveratrol required only ERα. A somewhat

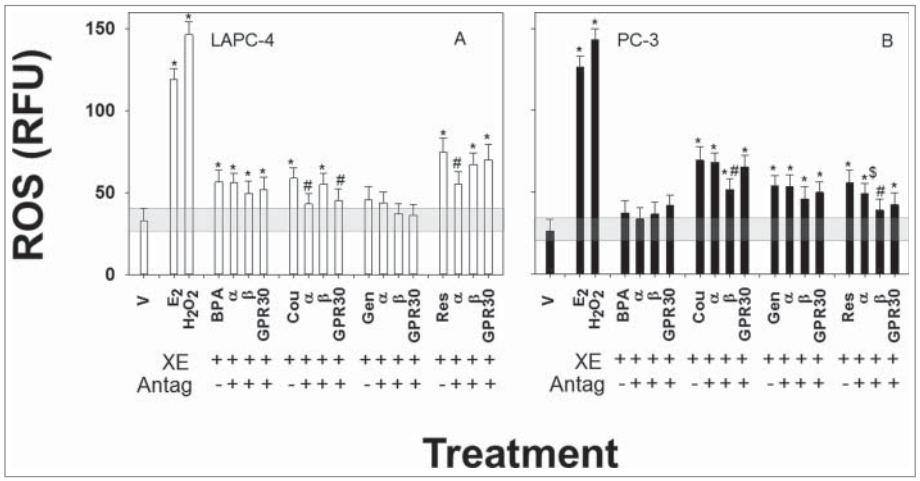

Figure 4. ROS levels after treatment with 10^{-10}M E_2, 10^{-6}MH_2O_2, 10^{-9}MBPA, 10^{-7}M coumestrol, 10^{-7}M genistein, and 10^{-8}M resveratrol, ± ER subtype-selective antagonists. Antagonists (Antag) were 10^{-7}M MMP for ERα; 10^{-6}M PHTPP for ERβ; and 10^{-6}M G15 for GPR30. ROS levels were measured after 15 min of each XE treatment (the optimal response time). *denotes significance from vehicle (V) controls at $P < 0.05$, while # denotes significance from paired XE treatment values ($P < 0.05$). ERα inhibition was significantly different vs. resveratrol alone in PC-3 cells ($^{\$}$) at $P < 0.09$. The shaded horizontal bars represent the response to vehicle (V) ± SEM.

different profile was evident in PC-3 (late-stage) cells, where coumestrol and resveratrol both increased ROS via ERβ, while genistein stimulation of ROS did not require any known ERs, and BPA did not cause a response (**Fig. 4B**). Clearly, the regulation of this pathway via ERs became quite different as cell types progressed to a less differentiated state with far lower receptor numbers (**Fig. 2**). The involvement of more than one ER subtype in some XE-generated responses also suggests the participation of multiple pathways.

Total and phosphorylated cyclin D1

Control of cyclin D1 levels[102,103] is a mechanism we previously identified as being a significant contributor to E_2- or DES-evoked declines in prostate cancer cell survival.[69] Both of these estrogens caused rapid cyclin D1 phospho-activation leading to swift degradation of this cell-cycle protein in early-stage cells, while this mechanism only operated for E_2 in late stage cells. Lower cyclin D1 levels was reflected also in lower cell numbers. Similar responses to single effective concentrations of XEs (changes in phosphorylation of cyclin D1; changes in total cyclin levels) are shown in **Fig. 5**. Of the 4 XEs studied here, the 3 phytoestrogens (coumestrol, genistein, and resveratrol) all affected cyclin D1 phosphorylation levels, though in distinctly different directions, and differently for early- vs. late-stage cells (**Figs. 5A & B**). In each case where cyclin D1 was phosphorylated, the corresponding expected rapid decline in total cyclin D1 levels occurred (panels C and D). Interestingly, we observed significant declines in total cyclin D1 as early as 5 min after genistein or resveratrol treatment (~10%), but the largest decreases for most compounds were seen after 4 h. Coumestrol caused cyclin dephosphorylation, resulting in cyclin D1 level increases, correlating very well with its ability to increase cell numbers. Resveratrol signaling significantly phosphorylated cyclin D1 in both cell lines, driving total cyclin D1 levels down, yet while eliciting very small increases in cell proliferation, a less perfect correlation. Genistein was the only compound that caused opposing effects on these mechanisms in the 2 cell lines. In LAPC-4 cells, it increased phosphorylated cyclin D1, causing its degradation, but that did not correlate with measured changes in viable cell numbers. However, in PC-3 cells, genistein depressed cyclin phosphorylation, allowing increases in cyclin levels and correlating with a strikingly robust cell proliferative response. BPA did not affect cyclin D1 phosphorylation in either cell line, nor did it change levels of total cyclin D1, in keeping with its minimal effects on cell numbers. While these correlations generally go in the expected direction, they do not entirely predict the degree of the functional (cell number-changing) responses. Therefore, we ultimately considered all of these mechanisms together (see summation in **Fig. 6**), to see if other mechanisms in some cases modified this dominant response to cyclin D1 changes (see Conclusions). Others have also shown that phytoestrogens can affect other cell-cycle protein levels,[26,27,31,32,104] which in turn affected the number of cells.

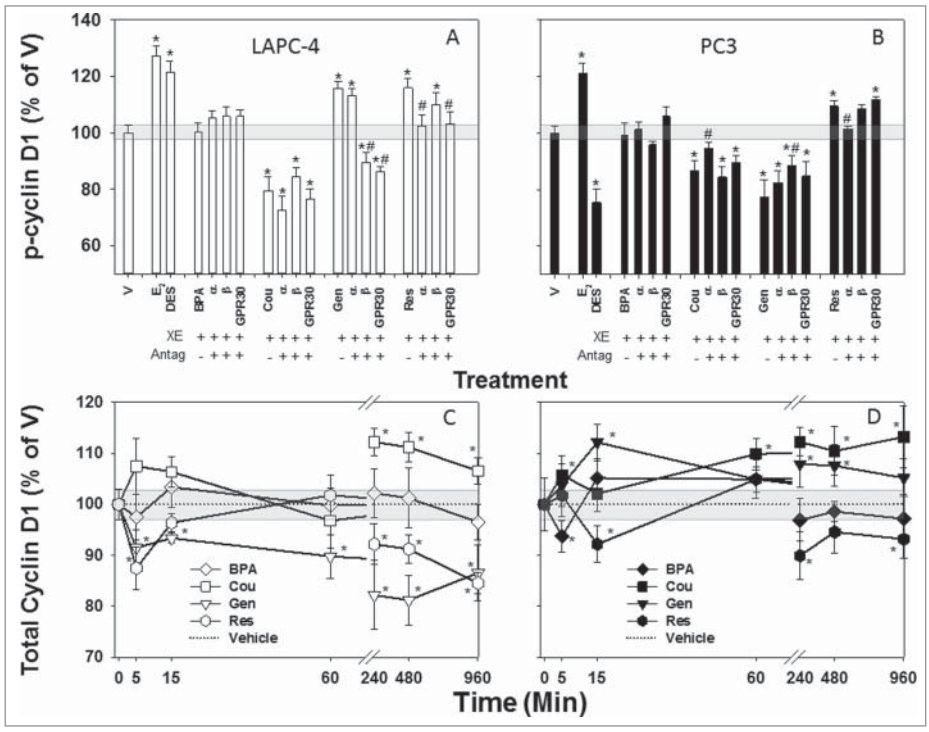

Figure 5. Cyclin D1 phosphorylation and degradation by XEs, and inhibition by ER-selective antagonists. Cyclin phosphorylation was measured at 1–60 min, and total cyclin D1 levels over 16 h of XE treatment. For 5A and 5B, LAPC-4 and PC-3 cells were pretreated with antagonists (Antag) for each of the 3 ER subtypes: α (MPP), β (PHTPP), and GPR30 (G15,) and then treated with 10^{-9}M BPA, 10^{-7}M coumestrol, 10^{-7}M genistein, 10^{-8}M resveratrol, 10^{-10}M E_2 or 10^{-6}M DES. Shaded horizontal bars represent V ± SEM. * denotes significance compared to vehicle (V) at $P < 0.05$. # denotes significance from paired XE treatment responses at $P < 0.05$. For 5C and 5D, LAPC-4 and PC-3 cells were treated with each XE for the times indicated and total cyclin D1 levels were measured with a plate immunoassay.

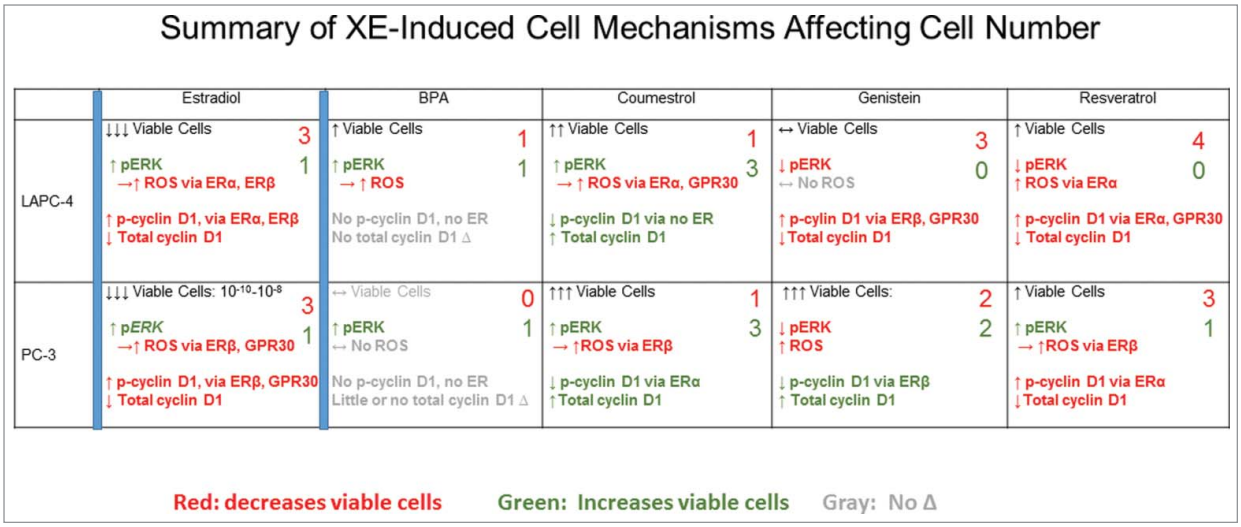

Figure 6. Summary of XE responses for mechanisms that affect the number of viable LAPC-4 vs. PC-3 cells. Estradiol is shown for comparison, summarizing the data from our previous publication [69]. Mechanisms in red text contribute to decreases in viable cell numbers, while mechanisms in green text increase the number of viable cells. Gray text indicates mechanisms that did not make any contribution to changes in cell numbers. These mechanistic contributions are summed in the red and green numbers in the upper right-hand corner of each box. Δ = change

degradation.[107] Other pathways that can contribute to cyclin D1 degradation include p38, which we previously studied for activation by DES and E_2 [69] and did not include here because it was activated for all cell types and treatments (and therefore may be permissive, but not directly causative). Cyclin D1 phosphorylation through p-p38 seems to be especially prevalent in response to the damage to DNA caused by environmental agents which require rapid cellular responses to prevent propagation of genomic mistakes.[108,109] The Mirk/Dyrk1b kinase, active during G0/G1, has also been shown to regulate cyclin D1 protein levels through phosphorylation at Thr288.[110] Therefore, multiple pathways can cause phosphorylation of cyclin D1, but they may influence degradation of cyclin D1 to varying degrees, and may be tissue-selective.

We next examined which ER subtypes (α, β, or GPR30) might be involved in the cyclin D1 phosphorylations, again using selective antagonists for each receptor subtype. For genistein, either the ERβ or GPR30 antagonist reversed the cyclin D1 phosphorylation in LAPC-4 cells, but only the ERβ antagonist decreased p-cyclin D1 levels in PC-3 cells. Coumestrol apparently did not utilize any of the known ER subtypes in LAPC-4 cells to decrease p-cyclin D1 levels, but ERα was required in PC-3 cells. Coumestrol's lack of dependence on any known receptor subtype in LAPC-4 cells is surprising, given the plentiful expression of all of these receptors in that cell line. It is possible that the low p-cyclin levels and thus larger errors in the measurement, caused by coumestrol treatment made antagonist reversals difficult to detect. Resveratrol's induction of p-cyclin D1 levels in both cell lines showed a dependence on ERs α and GPR30 in LAPC-4 cells, and on ERα in PC-3 cells. Therefore, each XE showed a dependence on a different ER subtype or subtype combination in the 2 cell lines. These dependencies are consistent with what has been previously shown about receptor subtype binding preferences for these XE compounds.[6] For example, resveratrol has a higher binding preference for ERα than for ERβ,[2] while coumestrol and genistein are strong ERβ agonists,[3] but are still capable of binding to ERα.[6,111,112] The predominance of ERβ in these prostate cell lines may influence their responses to these XEs that affect cell number. Mostly genomic pathways have been examined in the past, such as the ability to activate ER reporter constructs, with differences between cell types for different XEs.[113-115] Few comparisons for nongenomic responses are available, though we previously observed different MAPK activation patterns and mostly positive proliferative responses to E_2 and various XEs in GH3/B6/F10 pituitary cells[116] that have high mERα and low mERβ levels.

Also consistent with our results are known XE effects that do not involve these ERs. An example is the well-known direct inhibitory effect of genistein on tyrosine kinases.[117-119] In other instances, small lipophilic compounds like these can intercalate into cell membranes and as "border lipids" influence the actions of proteins embedded in them. Lipophilic estrogens can change membrane fluidity,[120,121] especially when they are present at relatively high concentrations (as is true for most effective phytoestrogen concentrations resulting from dietary exposures). Because changes in cell numbers are best observed after 3 d, the nuclear-localized receptors forms involved in slower transcriptional regulation[122,123] may also be relevant to these effects, which we did not examine in our studies of these more novel rapid actions.

Another possible contributor to tumor cell behavior in prostate cell lines is the tumor-suppressor p53.[124] It is mutated in LAPC-4 cells, and not present in PC-3 cells, and therefore unlikely to drive estrogen-mediated prostate tumor viability in our present studies. We also chose cell models for our studies that do not present the added complication of mutant ARs (such as in LnCaP cells[125]) to which estrogens can more readily bind and elicit effects, especially at high

concentrations. LAPC-4 cells have wild-type ARs and PC-3 cells do not express ARs.[124]

Conclusions

The estrogen-induced mechanism that dominated our effective predictions of cell growth behavior in these studies was the rapid phosphorylation of cyclin D1, followed shortly thereafter by its degradation. This mechanism largely predicted the primary response to each XE in terms of cell number changes (except in the case of resveratrol). However, no single mechanism entirely predicted the degree of these XE-induced changes, so we also examined the balance of the effects of other pathways (summarized in **Fig 6**). In this figure, each of the mechanisms examined in these studies for each cell type due to each XE is summarized in colored text: red denotes actions causing decreases in viable cells, while green represents mechanisms that increase cell numbers, and gray indicates no effect. These mechanistic contributions are summed in the red and green numbers in the upper right-hand corner of each box. The modest effects of these XEs are in contrast to the strong cell growth inhibitory/cell killing effects we saw previously with E_2; these previous conclusions [69] are shown in the first column of the table for the sake of comparison. E_2 engaged a total of 7 signaling responses in LAPC-4 cells and PC-3 cells – though in this table we list only the 4 that were examined here that gave the best predictions of therapeutic responses (decreases in cell numbers) to compare with XEs. Coumestrol is a robust stimulator of cell growth in both early- and late-stage prostate cancer cells, matching its strong positive effects on cyclin D1 status and levels. The ability to activate sustained ERK, and through it to increase ROS levels, did not decrease the cyclin D1-driven outcome. Genistein's different effects on early- vs. late-stage cells could also be largely explained by the cyclin D1 changes. Resveratrol had a very small effect in both cell lines, though the altered cyclin D1 levels and the ROS generation should have predicted a large decrease in cell viability, which was not observed. BPA showed the smallest changes in these responses that we examined, corresponding to only minimal growth stimulation, in only LAPC-4 cells.

Once again, mechanistic responses (phosphorylations, cyclin level changes, and ROS generation) to these varied estrogens were documented to be very rapid, supporting the notion that important tumor-altering effects can occur, or at least be initiated, via nongenomic signaling mechanisms. This was supported by our demonstration that membrane versions of these receptors are present in these tumor cells. Our present and recent [20,37,38,40,54,55,64,67,126] studies continue to support conclusions about the ability of XEs to imperfectly mimic physiological and pharmaceutical estrogens, as well as their unique patterns of mechanism engagement and ER requirements.[20,37,38,54,55,64,67,87,126,127] Because of these differences, it is important to consider the potential effects of each XE individually at its typical culturally- or environmentally-relevant levels, to determine what exposure advice these mechanistic studies may point to. Given that some of these compounds can have profound stimulatory effects on prostate cancer cell numbers (particularly the soy-related phytoestrogens coumestrol and genistein, and especially in late-stage tumor cells), it may be prudent to advise such patients against consuming foods that contain these phytoestrogens. On a lower priority level, resveratrol and BPA exposures may warrant similar warnings (**Fig 6**). Because BPA and genistein had different effects on the number of viable cancer cells, depending on prostate cancer stage, patients having recurring or long-term tumors may need different exposure advice. Because cell growth-promoting mechanisms receive stimulation via different ER subtypes depending upon the compound, it may be prudent to recommend blocking of these effects via all of these receptors. Alternatively, testing of a patient's individual tumor receptor profiles may allow for tailoring of therapies with antagonists for individual receptor subtypes.

Our initial hypothesis was that some of these alternative dietary estrogens might fulfill the hoped for anti-tumor signaling and cell growth effects. It appears that this is not the case, and the best estrogen to mediate tumor cell killing effects is E_2, profiled in more detail for these mechanisms (and others) in our previous report.[69] Taken together, these findings should have profound implications for dietary recommendations for prostate cancer patients, as well for as the development of ER-specific treatments to shrink tumors or slow tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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