Increased consumption of soy and soy isoflavones is associated with a reduced risk for prostate cancer (PCa). PCa progression is characterized, in part, by a loss of luminal/basal epithelial differentiation; however, the effects of soy isoflavones on cellular differentiation in the prostate are unknown. The present study examined the effects of the soy isoflavone glycitein on cellular differentiation in prostate epithelial cells (RWPE-1, WPE1-NB14, and RWPE-2). Glycitein significantly inhibited RWPE-1 cellular proliferation at concentrations ranging from 0.4 to 50 µM. Expression of the luminal epithelial cell marker cytokeratin 18 was not affected by glycitein treatment in the WPE1-NB14 and RWPE-2 cell lines. However, expression of cytokeratin 18 and prostate specific antigen (PSA) was decreased in the RWPE-1 cell line in response to glycitein treatment, whereas the expression of the basal epithelial cell markers p63 and cytokeratin 5 remained unchanged. These data suggest that glycitein may induce basal cell differentiation in the RWPE-1 cell line.

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer-related deaths among American males (1). Although the risk for cancer is multifactorial, substantial portions of cancer incidence rates are related to environmental factors including diet. Asian populations have lower PCa incidence and mortality as compared to the United States (2–5). The increased consumption of soy and soy-containing products within these populations may contribute to reduced cancer rates. However, the specific compounds in soy and their mechanisms of action in the prostate are unknown.

Prostate carcinogenesis is characterized as a continuum of impairment of the homeostatic control governing differentiation, proliferation, and apoptosis of the prostate epithelium. The prostate epithelium consists of 2 primary differentiated cell types, luminal and basal, which are characterized primarily by their unique cytokeratin profiles. Loss of luminal cell differentiation and a concomitant increase in the proliferation of this cell type is initially observed in low-grade prostatic intraepithelial neoplasia (LGPIN) (6–8). Progression to high-grade PIN (HG-PIN) involves disruption and partial loss of the basal cell population. A complete loss of the basal cell population, increased proliferation, decreased apoptosis, and subsequent cancer cell invasion of the basement membrane, stroma, and surrounding tissues is characteristic of PCa. Interestingly, populations with high soy consumption have a reduced risk for HG-PIN and PCa development; however, the incidence of LGPIN is similar to those populations with low soy consumption (9,10). This suggests that soy consumption may reduce PCa incidence by maintaining the differentiation state of the prostate epithelium; however, this hypothesis has yet to be tested.

Soy isoflavones have been shown to induce cellular differentiation in many tissues (11–13); however, isoflavone-induced differentiation has not yet been examined in the prostate. The objective of this study was to identify the potential of soy isoflavones to induce cellular differentiation of a prostate intermediate cell population.

MATERIALS AND METHODS

Materials

Genistein, daidzein, and equol were obtained from LC Laboratories (Woburn, MA). Glycitein was purchased from Indofine (Hillsboro, NJ). N-(4-hydroxyphenyl) retinamide (4-HPR) was obtained from Tocris (Ballwin, MO). The above compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and were utilized in cell culture media at appropriate dilutions, with a final DMSO concentration of 0.1%. Bicinchoninic acid (BCA) protein assay kit and superSignal ECL chemiluminescent substrate kit were obtained from Pierce (Rockford, IL). ECL Western blotting detection reagent was obtained from Amersham Biosciences Corporation (Piscataway, NJ). The monoclonal antibodies for cytokeratin 8/18 and β actin were obtained from Cell Signaling-Technology (Beverly, MA). The polyclonal antibodies for PSA, vimentin, cytokeratin 5, and p63 were obtained from Abcam Inc. (Cambridge, MA).
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). DNA flow cytometry analysis kit was purchased from Roche Applied Science (Indianapolis, IN).

**Cell Culture**

The human prostate epithelial cell lines RWPE-1, WPE1-NB14, and RWPE-2 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in keratinocyte serum-free medium (GIBCO Laboratories, Grand Island, NY) supplemented with 50 µg/ml bovine pituitary extract, 5% l-glutamine, and 5 ng/ml epidermal growth factor (EGF). These cells were maintained in a humidified incubator (5% CO₂, 95% O₂) at 37°C.

**Prostate Epithelial Proliferation**

RWPE-1 cells were plated in 48-well plates at an initial density of 1.0 × 10⁵ cells per well with supplements. Cells were treated with or without genistein, daidzein, equol, and glycitein alone and in combination at appropriate concentrations for 0 to 8 days, changing media with treatment every 48 h. After incubation, cell proliferation was determined by the MTT assay as described previously (14) and quantified spectrophotometrically at 595 nm.

**Immunoblot Analysis**

RWPE-1, WPE1-NB14, and RWPE-2 cells were plated in 60 mm dishes and treated with or without genistein, daidzein, equol (50 µM), glycitein (5 and 50 µM), or 4-HPR (1 µM) for 8 days, changing media every 48 h. Following treatment, cells were washed with PBS and crude proteins isolated and separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblot was performed using primary antibodies (cytokeratin 8/18, cytokeratin 5/14, p63, PSA, vimentin) at recommended dilutions in 1× PBS with 0.1% Tween-20) overnight at 4°C. Following incubation with secondary antibodies, protein signals were visualized on autoradiography film and quantified by densitometry using Scion imaging software (Frederick, MD).

**Morphology**

RWPE-1 cells were plated in a 4-well chamber slide and treated with or without glycitein (5 and 50 µM) or 4-HPR (1 µM) for 8 days. After treatment, cells were washed with PBS and fixed with 1% glutaraldehyde for 30 min at 4°C. Following fixation, cells were washed with PBS and stained with 0.2% crystal violet for 2 h at room temperature as previously described (15). Cells were then washed with PBS and examined with Olympus IX50 (B&B Microscopes Ltd., Pittsburgh, PA) inverted fluorescent microscope fitted with appropriate filter cubes.

**Cell Cycle Analysis**

RWPE-1 cells were plated in 100 mm dishes and treated with or without glycitein (5 and 50 µM) or 4-HPR (1 µM) for 3 days. After treatment, cells were washed with PBS, trypsinized, and fixed in −20°C ethanol at 4°C for 30 min. Cells were washed twice with PBS. DNA content was determined using the cellular DNA flow cytometry analysis kit (Roche Applied Science). Briefly, the samples were incubated at 37°C with RNase A for 30 min and treated with propidium iodide at 4°C for 2 h. A minimum of 20,000 events per sample was measured for DNA content by propidium iodide staining using the BD FACS calibur flow cytometer, and cell cycle distribution was determined using the software program Modfit (Verity Software House, Topsham, ME).

**Statistical Analysis**

Statistical significance between groups for proliferation, cell cycle, and immunoblot was determined with either 1-way analysis of variance (ANOVA) with Tukey’s post hoc comparisons or 2-sample t-test adjusted for multiple comparisons (SigmaStat software, Chicago, IL). Data are presented as means ± standard error of the mean with alpha P < 0.05 considered significant.

**RESULTS**

**RWPE-1 Cellular Proliferation**

The antiproliferative effects of genistein, daidzein, equol, and glycitein on RWPE-1 cells is given in Figs. 1A to 1D. RWPE-1 cellular proliferation was significantly reduced on treatment with 50 µM of genistein, daidzein, equol, and glycitein by 88.35 ± 8.29%, 29.43 ± 13.73%, 69.21 ± 6.82%, and 84.62 ± 7.88%, respectively, compared to untreated controls (P < 0.001). A 19.4 ± 1.1% increase in cellular proliferation was observed after treatment with 5 µM genistein. Glycitein significantly reduced RWPE-1 proliferation by 21.20 ± 3.01% at concentrations as low as 5 µM compared to untreated controls (P < 0.01).

The cytotoxic effects of genistein and glycitein at 50 µM were measured and are given in Fig. 2A. After 8 days of treatment, glycitein did not alter the concentration of the initial cell population measured at Day 0. Genistein significantly reduced the initial cell population by 25.2 ± 8.30% (P < 0.05).

The antiproliferative effects of isoflavones in combination and glycitein alone are given in Figs. 2B and 2C. Cells were treated with 0.5 µM genistein, 2.8 µM daidzein, 2.7 µM equol, and a range of concentrations of glycitein (0–50 µM) for 8 days, and cellular proliferation was measured (Fig. 2B). Combinations of genistein, daidzein, and equol did not effect the proliferation of the RWPE-1 cell line. However, when glycitein (10 and 50 µM) was added to the isoflavone cocktail, proliferation was reduced 31.7 ± 3.1% and 72.4 ± 7.2%, respectively (P < 0.05). Glycitein treatment alone significantly reduced the proliferation of this cell line at all concentrations tested (P < 0.05; Fig. 2C).
Expression of Luminal and Basal Epithelial Cell Markers

Effect of isoflavones (50 µM) on the expression of cytokeratin 18, a marker of luminal cell differentiation, is given in Fig. 3A. Genistein, daidzein, and equol induced expression of cytokeratin 18. However, glycitein reduced the expression of cytokeratin 18. Therefore, the effect of glycitein on cellular differentiation was further characterized.

Treatment with 50 µM glycitein significantly reduced expression of cytokeratin 18, 8 days posttreatment (P < 0.001; Fig. 3B). 4-HPR (1 µM), a known inducer of luminal differentiation, was used as a control and significantly increased expression of cytokeratin 18 (P < 0.005). Glycitein did not affect the expression of cytokeratin 18 in the WPE1-NB14 and RWPE-2 cell lines (Fig. 3C).

The effect of glycitein on basal cell protein marker expression is given in Fig 4. Glycitein (5 and 50 µM) maintained expression of cytokeratin 5 (Fig. 4A) and p63 (Fig. 4B). 4-HPR (1 µM) significantly decreased expression of these basal cell makers.

RWPE-1 Epithelial Cell Markers

To confirm that glycitein-treated cells remained epithelial in origin, expression of vimentin was measured and is given in Fig. 5A. Expression of vimentin remained undetected following 8 days treatment with 4-HPR (1 µM) and glycitein (5 and 50 µM) compared with untreated controls. Primary dog lens epithelial cell lysate collected at passage 2 was used as the positive control (16). Furthermore, gross morphology of cells treated with 4-HPR and glycitein resemble untreated controls (Fig. 5B).

RWPE-1 Cell Cycle Distribution

Effects of glycitein on cell cycle distribution are given in Table 1. Glycitein did not significantly alter cell cycle distribution at 5 µM. However, at 50 µM, glycitein significantly increased the amount of cells in the G2-M phase by 5.8 ± 0.7% and decreased S phase by 5.1 ± 0.9% (P < 0.05). Although these results were statistically significant, glycitein did not appreciably alter cell cycle distribution. 4-HPR (1 µM), a synthetic retinoid and known inducer of G0-G1 cell cycle arrest, was used as a positive control.

DISCUSSION

The objective of this study was to examine the effects of soy isoflavones, specifically glycitein, on prostate epithelial differentiation. It has been hypothesized that sustained extracellular signal-regulated kinase (ERK1/2) signaling is involved the differentiation process in the prostate epithelium (17). Previous work in this laboratory demonstrates that glycitein induces a
sustained and robust activation of the ERK1/2 signaling cascade in the RWPE-1 cell line (18). Based on this observation, we hypothesize that soy isoflavones may influence the differentiation of this cell type. We tested this hypothesis using RWPE-1 cells as a model for prostate epithelial cell differentiation. This cell line expresses both luminal and basal cell protein markers and is therefore considered an intermediate prostate epithelial cell type. Research supports the differentiation of this intermediate cell type into either luminal or basal cells during organogenesis (19). Dysregulated prostate epithelial cell differentiation is commonly observed during prostate carcinogenesis. Therefore, strategies to maintain proper prostate cell differentiation may be beneficial in the treatment and/or prevention of PCa.

FIG. 2. Antiproliferative effects of isoflavones alone and in combination (A). RWPE-1 cells were treated for 0 or 8 days with 50 µM genistein or glycitein. Cellular proliferation was assessed at Day 0 and Day 8. Concentration dependent effects of glycitein (0–50 µM) (Fig. 2B) in combination with physiologically relevant concentrations of genistein (0.5 µM), daidzein (Daid), equol, glycitein (Gly), or vehicle alone (Con), changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot. B: Expression of cytokeratin 18 was measured by immunoblot in RWPE-1 cells following 8 days treatment with 4-HPR (1 µM), glycitein (5 or 50 µM), or vehicle alone, changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot and quantified. β-actin was used as the loading control. Data are given by percent of means ± SEM. Mean values not sharing common letter superscripts differ significantly (P < 0.05). C: Expression of cytokeratin 18 was measured by immunoblot in WPE1-NB14 and RWPE-2 cells following 8 days treatment with glycitein (50 µM) or vehicle alone, changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot. All experiments were performed independently 3 times with n = 3.

FIG. 3. Effect of isoflavones (50 µM) on expression of luminal cell differentiation proteins. A: Expression of cytokeratin 18 was measured by immunoblot in RWPE-1 cells following 8 days treatment with 50 µM genistein (Gen), daidzein (Daid), equol, glycitein (Gly), or vehicle alone (Con), changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot. B: Expression of cytokeratin 18 was measured by immunoblot in RWPE-1 cells following 8 days treatment with 4-HPR (1 µM), glycitein (5 or 50 µM), or vehicle alone, changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot and quantified. β-actin was used as the loading control. Data are given by percent of means ± SEM. Mean values not sharing common letter superscripts differ significantly (P < 0.05). C: Expression of cytokeratin 18 was measured by immunoblot in WPE1-NB14 and RWPE-2 cells following 8 days treatment with glycitein (50 µM) or vehicle alone, changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot. All experiments were performed independently 3 times with n = 3.
FIG. 4. Effect of glycitein on expression of basal cell differentiation proteins. A: Expression of cytokeratin 5 was measured by immunoblot in RWPE-1 cells following 8 days treatment with 4-HPR (1 µM), glycitein (5 or 50 µM), or vehicle alone, changing media every 48 h. β-actin was used as the loading control. B: Expression of p63 was measured by immunoblot in RWPE-1 cells following 8 days treatment with 4-HPR (1 µM), glycitein (5 or 50 µM), or vehicle alone, changing media every 48 h. β-actin was used as the loading control. Data are given as a representative immunoblot. Data are given as a representative immunoblot. All experiments were performed independently 3 times with \( n = 3 \). Significant difference from the untreated control is designated by letter superscripts (\( P < 0.05 \)).

The soy isoflavones genistein, daidzein, and the daidzein metabolite equol have been extensively studied for their anti-cancer properties in several in vitro and in vivo models (20–31). However, limited data are available regarding the anticancer effects of glycitein. Glycitein has been shown to reduce cellular invasion (32,33) and cell motility (34) in Jurkat T and breast cancer cell lines. In prostate cell lines, glycitein has been shown to induce cell cycle arrest (35), decrease cellular proliferation (18,35), and modulate signal transduction pathways (18). The present study examined the antiproliferative effects of genistein, daidzein, equol, and glycitein and found that all isoflavones inhibited proliferation of the nontumorigenic prostate epithelial cell line, RWPE-1, at 50 µM. Genistein and glycitein were the most potent inhibitors of cell proliferation at the highest concentration tested. The decrease in cellular proliferation by genistein appears to be cytotoxic, whereas the decrease in cellular proliferation by glycitein appears to be cytostatic. Previous studies in

![Flow cytometric analysis of RWPE-1 cells treated with 4-HPR (1 µM), glycitein (50 µM), or untreated control for 3 days](image)

**TABLE 1**

Flow cytometric analysis of RWPE-1 cells treated with 4-HPR (1 µM), glycitein (50 µM), or untreated control for 3 days

<table>
<thead>
<tr>
<th>Cell Cycle Distribution</th>
<th>Control</th>
<th>4-HPR</th>
<th>5 µM Glycitein</th>
<th>50 µM Glycitein</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₀-G₁</td>
<td>63.6(_a) ± 0.8</td>
<td>71.5(_b) ± 2.7</td>
<td>65.9(_a) ± 1</td>
<td>62.9(_a) ± 1.2</td>
</tr>
<tr>
<td>S</td>
<td>29.2(_a) ± 2.8</td>
<td>12.8(_b) ± 0.6</td>
<td>26.2(_ab) ± 1.2</td>
<td>24.1(_b) ± 0.9</td>
</tr>
<tr>
<td>G₂-M</td>
<td>7.2(_a) ± 1.9</td>
<td>15.8(_b) ± 2.2</td>
<td>7.9(_a) ± 0.2</td>
<td>13.0(_b) ± 0.7</td>
</tr>
</tbody>
</table>

\(^a\)Abbreviation is as follows: 4-HPR, N-(4-hydroxyphenyl) retinamide. All experiments were performed independently at least 3 times with \( n = 3 \). Significant difference from the untreated control is designated by letter subscripts (\( P < 0.05 \)).
this laboratory support these findings that genistein reduces cell proliferation at >12.5 \( \mu M \), primarily via apoptosis (14).

Glycitein inhibited RWPE-1 proliferation at concentrations 10-fold less than the other isoflavones. This observation may be attributed to the methoxy group on the sixth carbon of the flavone ring of glycitein. Recent studies suggest that flavones with methoxy groups are more biologically stable, resistant to metabolism, and have improved intestinal transport as compared to their nonmethylated counterparts (36,37).

The concentration of genistein, daidzein, and equol in prostatic fluid has previously been reported to be an average of 0.5, 2.8, and 2.7 \( \mu M \), respectively (38). To date, the concentration of glycitein in prostatic fluid has not been reported. Glycitein alone reduced RWPE-1 cell proliferation at all concentrations tested (0–50 \( \mu M \)). Interestingly, when glycitein was given in combination with prostatic fluid concentrations of genistein, daidzein, and equol, the antiproliferative effect of glycitein was reduced. This suggests a possible antagonism between isoflavones. It has been suggested that the antagonistic effects between soy isoflavones may be due to receptor site competition (39). However, further research is necessary to explore synergistic and antagonistic effects of combinations of physiologically relevant soy isoflavones in the prostate.

The results of the present study show that glycitein altered the expression of specific protein markers consistent with cellular differentiation in the RWPE-1 cell line. Glycitein down-regulated the expression of luminal epithelial cell markers and maintained the expression of basal epithelial cell markers. However, glycitein did not alter expression of cellular differentiation markers in the WPE1-NB14 precancerous and RWPE-2 cancerous prostate cell lines, suggesting that the differentiation effects of glycitein may be cancer stage specific. We and others have previously shown that the bioactivity of dietary compounds may be most beneficial during specific stages of PCa and that the timing of exposure is critical for maximizing the anticancer effects (18,31,40–42). Furthermore, several epidemiological studies have suggested that a diet rich in soy and soy isoflavones may prevent PCa during noncancerous and precancerous stages of the carcinogenic process and that during advanced disease, exposure to soy isoflavones may no longer be beneficial (43–46).

To further support our hypothesis that glycitein induces basal cell differentiation in the RWPE-1 cell line, PSA levels were measured following treatment with the androgen DHT (data not shown). PSA is a serine protease that is expressed only by luminal and intermediate cells of the prostate. Therefore, PSA is an appropriate marker for distinguishing between luminal and/or intermediate cells from basal cells. The synthetic retinoid 4-HPR induces luminal cellular differentiation in the prostate (42) and therefore was used as a positive control for the expression of PSA. The present study found that DHT did not induce intracellular expression of PSA in glycitein treated cells. However, DHT treatment did induce expression of PSA in vehicle and 4-HPR treated cells. These results further support our observation that glycitein influences the differentiation of an intermediate to a basal cell type.

The process of differentiation involves inhibited proliferation and an exit from the cell cycle as measured by \( G_0-G_1 \) cell cycle arrest. These data show that the positive control, 4-HPR, but not glycitein induced cell cycle arrest at the \( G_0-G_1 \) phase in the RWPE-1 cell line. Glycitein has been shown to induce cell cycle arrest in the \( G_0-G_1 \) phase of the cell cycle in primary prostate cell lines (35). We hypothesize that \( G_0-G_1 \) arrest was not observed upon glycitein treatment because immortalization of the RWPE-1 cell line with the human papilloma virus 18 (HPV-18) produces a protein that binds retinoblastoma (pRb), the major \( G_0-G_1 \) cell cycle check point protein. Studies suggest that fractions of soy paste induce \( G_0-G_1 \) cell cycle arrest in breast cancer cells via activation of pRb (47) and that 4-HPR induced \( G_0-G_1 \) cell cycle arrest is independent of pRb activation (48). This may explain why 4-HPR but not glycitein induced \( G_0-G_1 \) cell cycle arrest in our immortalized prostate epithelial cell line; however, the effect of glycitein on pRb activation has yet to be tested.

Disruption and subsequent loss of basal cells within the prostate epithelium is the most prominent morphological feature observed in HGPIN and PCas. Results from the present study suggest that glycitein induces genotypic changes in the RWPE-1 cell line consistent with basal epithelial cell differentiation. We hypothesize that glycitein-induced basal cell differentiation may preserve the basal cell population within the prostate, thus representing a novel mechanism by which dietary soy reduces PCa incidence.

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REFERENCES


