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Glycitein activates extracellular signal-regulated kinase via vascular endothelial growth factor receptor signaling in nontumorigenic (RWPE-1) prostate epithelial cells

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Abstract

Increased consumption of soy is associated with a decreased risk for prostate cancer; however, the specific cellular mechanisms responsible for this anticancer activity are unknown. Dietary modulation of signaling cascades controlling cellular growth, proliferation and differentiation has emerged as a potential chemopreventive mechanism. The present study examined the effects of four soy isoflavones (genistein, daidzein, glycitein and equol) on extracellular signal-regulated kinase (ERK1/2) activity in a nontumorigenic prostate epithelial cell line (RWPE-1). All four isoflavones (10 μmol/L) significantly increased ERK1/2 activity in RWPE-1 cells, as determined by immunoblotting. Isoflavone-induced ERK1/2 activation was rapid and sustained for approximately 2 h posttreatment. Glycitein, the most potent activator of ERK1/2, decreased RWPE-1 cell proliferation by 40% ($P<.01$). Glycitein-induced ERK1/2 activation was dependent, in part, on tyrosine kinase activity associated with vascular endothelial growth factor receptor (VEGFR). The presence of both VEGFR1 and VEGFR2 in the RWPE-1 cell line was confirmed by immunocytochemistry. Treatment of RWPE-1 cells with $VEGF_{165}$ resulted in transient ERK1/2 activation and increased cellular proliferation. The ability of isoflavones to modulate ERK1/2 signaling cascade via VEGFR signaling in the prostate may be responsible, in part, for the anticancer activity of soy.
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Keywords: Prostate; Isoflavones; ERK1/2; Mitogen-activated protein kinases; VEGFR; Chemoprevention

1. Introduction

Prostate cancer is the second leading cause of cancer-related deaths in the United States [1]. Environmental factors, including diet, can influence the development of this disease. Both epidemiological and experimental evidence suggest that increased consumption of soy and soy-based foods is associated with a decreased risk for prostate cancer [2–5]. Soy isoflavones are thought to be responsible, in part, for this observed protective effect.

Soy isoflavones (Fig. 1) are a family of nonnutritive compounds that can modulate a variety of biological processes associated with carcinogenesis. The three most abundant isoflavones, genistein, daidzein and glycitein, comprise approximately 50%, 40% and 10% of the total soybean isoflavone profile, respectively. The majority of studies examining the anticancer effects of soy have focused on genistein. The anticancer effects of genistein include tyrosine kinase inhibition [6], topoisomerase inhibition [6], induction of cell cycle arrest [7–9], apoptosis [10] and increased antioxidant activity [11]. Genistein can also modulate growth factor signaling in the transgenic adenocarcinoma mouse prostate model [12] and can reduce the incidence and number of poorly differentiated tumors in vivo [13,14]. The biological effects of genistein, as well as of daidzein, appear to be primarily mediated via sex hormone (i.e., androgen and estrogen) signaling [15–20]. However, the specific cellular and molecular mechanisms by which isoflavones exert their anticancer activities are not well characterized.

The dietary and chemotherapeutic modulation of signal transduction cascades controlling cellular proliferation, apoptosis, differentiation and cell cycle progression has emerged as a potential therapeutic target for the treatment and prevention of prostate cancer. Extracellular signal-

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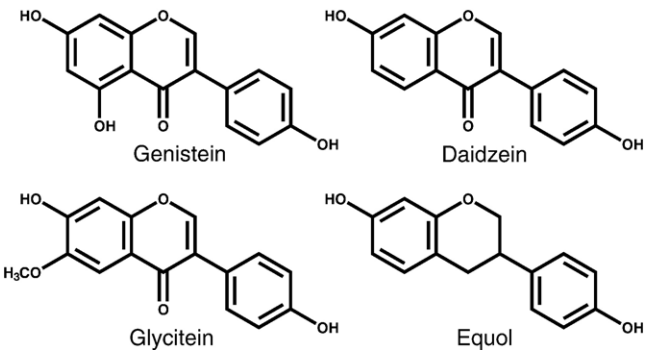

Fig. 1. Chemical structures of the principal soy isoflavones genistein, daidzein and glycitein, and the daidzein metabolite equol.

regulated kinase (ERK1/2) cascade is a member of the mitogen-activated protein kinase family and is required for the normal growth, development and survival of the prostate epithelium [21]. A number of recent studies have demonstrated that isoflavones, specifically genistein, modulate ERK1/2 activity in tumorigenic prostate cell lines [15,22,23]. The present study examined the effect of four soy isoflavones (genistein, daidzein, equol and glycitein) on ERK1/2 signaling in nontumorigenic prostate epithelial cells.

2. Materials and methods

2.1. Materials

Genistein, daidzein, glycitein, equol and the MEK inhibitor U0126 were obtained from LC Laboratories (Woburn, MA). Specific chemical inhibitors of tyrosine kinase activity for the following proteins — insulin-like growth factor receptor (IGFR), tumor growth factor β (TGFβ) receptor, steroid receptor coactivator (Src), vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) — were purchased from EMD Biosciences (La Jolla, CA). The above compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and were utilized in a cell culture medium at appropriate dilutions with a final DMSO concentration of 0.1%. The antiandrogen hydroxyflutamide (HF; Toronto Research Chemicals, North York, ON) and the antiestrogen ICI 182,780 (Tocris, Ballwin, MO) were dissolved in ethanol and diluted to a final ethanol concentration of 0.1%. Recombinant human $VEGF_{165}$ (R&D Systems, Inc., Minneapolis, MN) was dissolved in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin. Bicinchoninic acid 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). Polyclonal antibodies for phospho-ERK1/2 kinase and total ERK1/2 kinase were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies for VEGFR1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO) Vectastain avidin–biotin complex (ABC) kit was obtained from Vector Laboratories (Burlingame, CA).

2.2. Cell culture

The nontumorigenic human prostate epithelial cell line RWPE-1 and the metastasized human prostate cancer line PC-3 were purchased from the American Type Culture Collection (Rockville, MD). RWPE-1 cells were maintained in keratinocyte serum-free medium (KSFM; GIBCO Laboratories, Grand Island, NY) supplemented with 50 mg/L bovine pituitary extract, 5% L-glutamine and 5 μg/L EGF. The PC-3 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) were provided by Dr. Jim Waldman (Department of Pathology, The Ohio State University, Columbus, OH) and maintained in an endothelial cell growth medium consisting of M-199 medium, 20% FBS, 1 mol/L HEPES buffer, 0.6% bovine brain extract supplemented with heparin, 0.2% penicillin/streptomycin and 0.1% fungizone. All cell lines were maintained in a humidified incubator (5% CO_2, 95% O_2) at 37°C.

2.3. Immunoblot detection of active and total ERK1/2

RWPE-1 and PC-3 cells were grown in 60-mm dishes for 48 h (90% confluent). The growth medium was removed, and cells were washed with PBS and incubated in supplement-free medium for 24 h. Cells were then treated with isoflavones alone or in combination with specific inhibitors [U0126, HF, ICI 182,780 and chemical tyrosine kinase inhibitors (IGFR, TGFβ, Src, VEGFR, EGFR and PDGFR)] at appropriate times and concentrations. Following treatments, the growth medium was removed, and cells were washed with PBS. Crude proteins were isolated and separated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed overnight using primary antibodies that recognize active (phospho) and total ERK1/2 at recommended dilutions in TTBS (1×TBS with 0.1% Tween-20) at 4°C. Following incubation with secondary antibodies, protein signals were visualized on autoradiography film using ECL and quantified by densitometry using Scion imaging software (Scion, Frederick, MD). Basal levels of protein expression are given as 100%, and twice this level is reported as 200% of control.

2.4. Immunocytochemical detection of VEGFR1 and VEGFR2

RWPE-1 and HUVEC were grown in six-well plates until 50% confluent. The growth medium was removed, and cells were fixed with 4% paraformaldehyde at 4°C and permeabilized with 0.2% Triton X-100. Plates were then blocked with 5.5% normal goat serum and incubated with primary antibodies overnight at 4°C (1:50 anti-VEGFR1 and

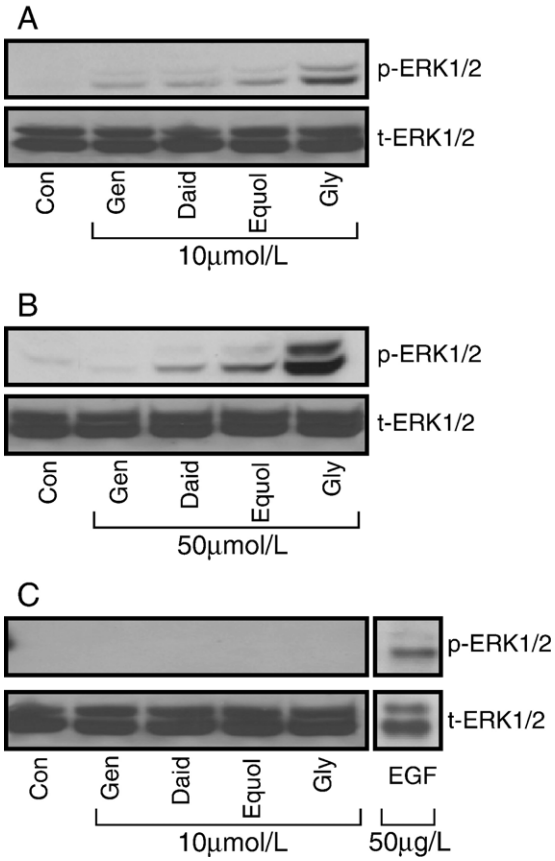

Fig. 2. Effect of isoflavones on ERK1/2 activity in nontumorigenic (RWPE-1) and tumorigenic (PC-3) prostate cells. Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in RWPE-1 cells following 2 h of treatment with either (A) 10 μmol/L or (B) 50 μmol/L genistein (Gen), daidzein (Daid), equol, glycitein (Gly) or vehicle alone (Con). (C) Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in PC-3 cells following either 2 h of treatment with 10 μmol/L genistein (Gen), daidzein (Daid), equol, glycitein (Gly) and vehicle alone (Con), or 10 min of treatment with 50 μg/L EGF. All experiments were performed independently at least thrice, with $n=3$ per experiment. Total ERK1/2 (t-ERK1/2) was used as loading control.

anti-VEGFR2). After incubation, samples were incubated with hydrogen peroxide (0.6%) to quench any endogenous peroxidase activity prior to secondary antibody (1:500). Signals were detected with ABC and diaminobenzidine reagent.

2.5. Cellular proliferation

RWPE-1 cells were plated with KSFM with supplements in 48-well plates at an initial density of 1.0×10^4 cells/well. Cells were treated with or without glycitein (50 μmol/L) or $VEGF_{165}$ (50 μg/L) alone or in combination with U0126 (10 μmol/L) and incubated for an additional 72 h. After incubation, cell proliferation was determined by MTT assay, as described previously [15]. Changes in cellular proliferation are expressed as percentages of control (given as 100%).

2.6. Statistical analysis

Statistical significance between groups was determined by one-way analysis of variance with Tukey's post hoc comparisons (SigmaStat software; SigmaStat, Chicago, IL). Data are presented as percentages of mean±relative standard error (RSE), with an α level of $P<.05$.

3. Results

3.1. Isoflavones induce ERK1/2 activity in RWPE-1 cells, but not in PC-3 cells

Isoflavone-induced cytotoxicity was not observed under any experimental condition (data not shown). Treatment of RWPE-1 cells with daidzein, equol and glycitein (10 and 50 μmol/L, 2 h) resulted in a concentration-dependent activation of ERK1/2 activity (Fig. 2A and B). As previously observed [15], treatment of RWPE-1 cells with genistein (2 h) at 10 μmol/L activated ERK1/2, while 50 μmol/L genistein inhibited ERK1/2. Glycitein was the most active ERK1/2 inducer at both 10 and 50 μmol/L (Fig. 2A and B). Treatment of PC-3 cells with genistein, daidzein, equol and glycitein (10 μmol/L) did not activate ERK1/2 (Fig. 2C). No changes in total ERK1/2 (t-ERK1/2) were observed with any treatment. The mechanism by which glycitein activates ERK1/2 was further characterized in the RWPE-1 cell line.

3.2. Hormone-independent ERK1/2 activation

We have previously shown that genistein induces ERK1/2 activity via an estrogen receptor-mediated mechanism in the RWPE-1 cell line [15]. In order to determine whether glycitein-induced ERK1/2 activity was also mediated via the estrogen receptor, RWPE-1 cells were treated with glycitein alone (50 μmol/L, 2 h) or in combination with the antiestrogen ICI 182,780 (10 μmol/L, 1 h pretreatment)

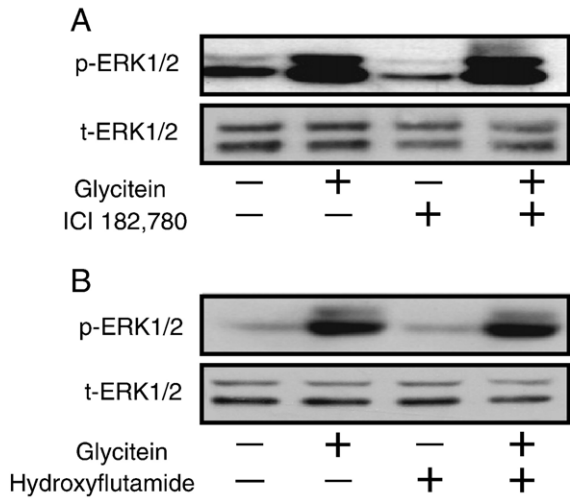

Fig. 3. Glycitein-induced ERK1/2 activity in RWPE-1 cells is independent of estrogen and androgen receptor activity. Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in RWPE-1 cells following treatment with or without (A) ICI 182,780 (antiestrogen) or (B) HF (antiandrogen) (10 μmol/L, 1 h) prior to glycitein treatment (50 μmol/L, 2 h). Data are given as representative immunoblots. All experiments were performed independently at least thrice, with $n=3$ per experiment. Total ERK1/2 (t-ERK1/2) was used as loading control.

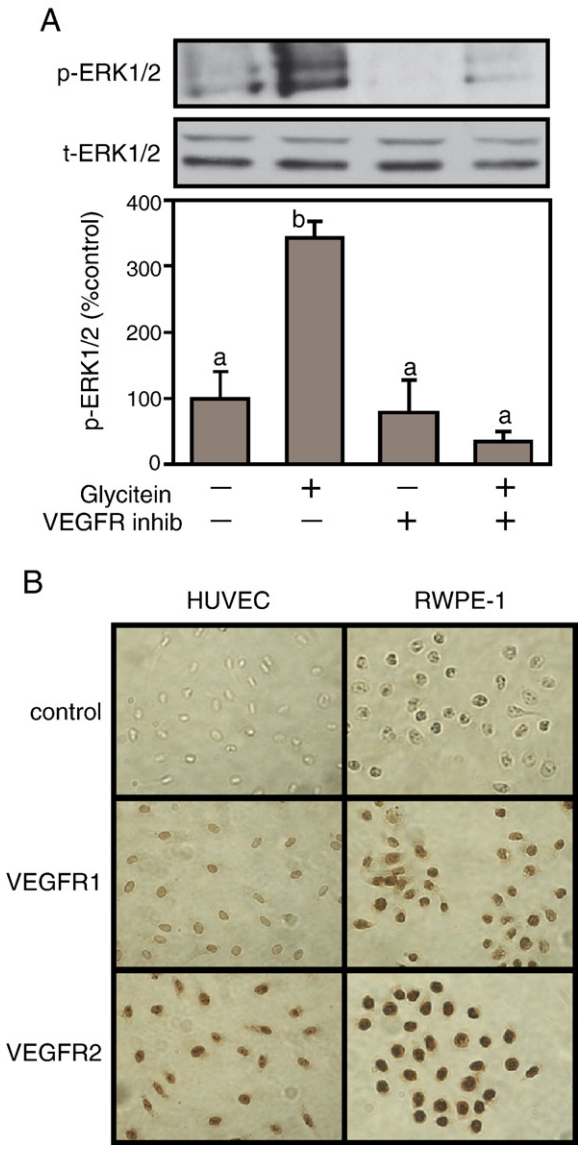

Fig. 4. Glycitein-induced ERK1/2 activity in RWPE-1 cells is dependent on VEGFR signaling. (A) Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in RWPE-1 cells following treatment with or without VEGFR tyrosine kinase inhibitor (0.10 μmol/L, 1 h) prior to glycitein (50 μmol/L, 2 h). All experiments were performed independently at least thrice, with $n=3$ per experiment. Data are presented as percentages of mean± RSE. Mean values not sharing a common superscript letter differ significantly ($P<.05$). Total ERK1/2 (t-ERK1/2) was used as loading control. (B) Immunocytochemical identification of VEGFR1 and VEGFR2 in RWPE-1 cells. The HUVEC line served as positive control for VEGFR1 and VEGFR2. Control treatments did not receive primary antibody.

(Fig. 3A). Glycitein treatment alone induced ERK1/2 activity, while ICI 182,780 treatment alone inhibited basal activity. Pretreatment with ICI 182,780 did not inhibit glycitein-induced ERK1/2 activity.

In order to determine whether glycitein-induced ERK1/2 activity was mediated via the androgen receptor, RWPE-1 cells were treated with glycitein alone (50 μmol/L, 2 h) or in combination with the antiandrogen HF (10 μmol/L, 1 h pretreatment) (Fig. 3B). Glycitein treatment alone significantly induced ERK1/2 activity ($P<.01$), while HF pretreatment did not alter basal ERK1/2 activity. Pretreatment with HF did not inhibit glycitein-induced ERK1/2 activation.

3.3. Glycitein activates ERK1/2 via a VEFGR-dependent mechanism

To identify the mechanism by which glycitein activates ERK1/2, RWPE-1 cells were pretreated (1 h) with several chemical tyrosine kinase inhibitors (IGFR, TGFβ, Src, VEGFR, EGFR and PDGFR) prior to glycitein (50 μmol/L, 2 h) treatment. Of those tested, only the VEGFR tyrosine

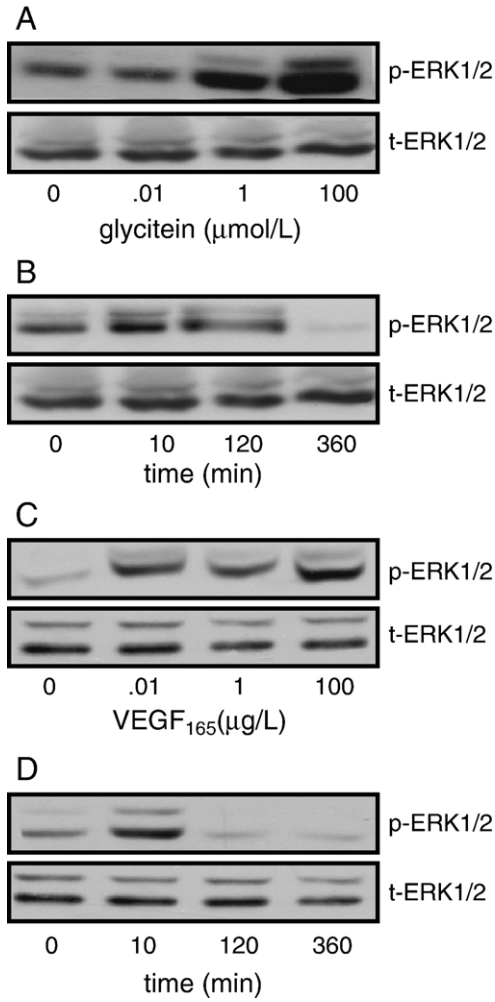

Fig. 5. Concentration-dependent and time-dependent induction of ERK1/2 activity by glycitein and $VEGF_{165}$ in RWPE-1 cells. (A) Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in RWPE-1 cells following 2 h of treatment with glycitein (0–100 μmol/L). (B) Time-dependent (0–360 min) activation of ERK1/2 following treatment of cells with 50 μmol/L glycitein. (C) Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in RWPE-1 cells following 10 min of treatment with $VEGF_{165}$ (0–100 μg/L). (D) Time-dependent (0–360 min) activation of ERK1/2 following treatment of cells with 50 μg/L $VEGF_{165}$. All experiments were performed independently at least thrice, with $n=3$ per experiment. Total ERK1/2 (t-ERK1/2) was used as loading control.

kinase inhibitor specifically inhibited glycitein-induced ERK1/2 activation ($P<.01$) (Fig. 4A).

Immunocytochemical analysis for VEGFR1 and VEGFR2 is presented in Fig. 4B. The expression of VEGFR1 and VEGFR2 was detected in both HUVEC (positive control) and RWPE-1. Samples receiving no VEGFR primary antibody served as negative control.

3.4. Concentration-dependent and time-dependent induction of ERK1/2 activity by glycitein and $VEGF_{165}$

Glycitein and $VEGF_{165}$-induced ERK1/2 activity in RWPE-1 cells was both concentration-dependent and time-dependent (Fig. 5A–D). Glycitein-induced ERK1/2 activation was observed at concentrations ranging from 0.01 to 100 μmol/L (Fig. 5A). ERK1/2 activity occurred within 10 min and remained active 2 h posttreatment. ERK1/2 activity fell below basal levels within 6 h posttreatment (Fig. 5B). $VEGF_{165}$-induced ERK1/2 activation was observed at concentrations ranging from 10 ng/L to 100 μg/L (Fig. 5C). $VEGF_{165}$-induced ERK1/2 activation peaked within 10 min (Fig. 5D) and returned to basal levels within 30 min (data not shown).

3.5. Effect of $VEGF_{165}$ and glycitein on cellular proliferation

The effects of $VEGF_{165}$ and glycitein-induced ERK1/2 activity on RWPE-1 cellular proliferation were determined using the MEK inhibitor, U0126. Treatment of RWPE-1 cells with glycitein (50 μmol/L) or U0126 (10 μmol/L) reduced cell proliferation by 40% and 45%, respectively ($P<.01$), 72 h posttreatment (Fig. 6A, left panel). When treated in combination, glycitein (50 μmol/L) and U0126 (10 μmol/L) reduced RWPE-1 cell proliferation by 50% at 72 h posttreatment ($P<.01$).

ERK1/2 activation in RWPE-1 cells following treatment with glycitein (50 μmol/L, 2 h) and U0126 (10 μmol/L, 1 h pretreatment) alone or in combination is presented in Fig. 6A (right panel). Glycitein treatment alone increased ERK1/2 activity, while pretreatment with U0126 inhibited both basal and glycitein-induced ERK1/2 activation.

The effect of $VEGF_{165}$ on RWPE-1 cellular proliferation is presented in Fig. 6B (left panel). Treatment of RWPE-1 cells with $VEGF_{165}$ (50 μg/L) increased cell proliferation by 23%, while treatment with U0126 (10 μmol/L) reduced cell proliferation by 30% at 72 h posttreatment ($P<.01$).

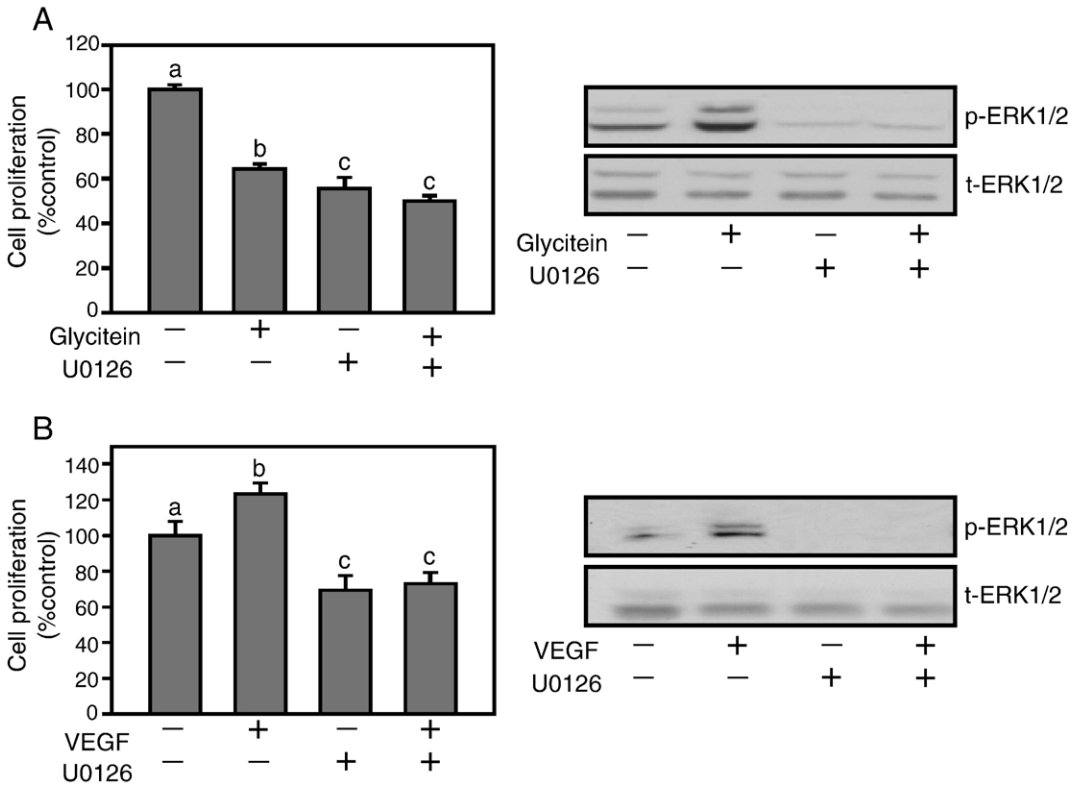

Fig. 6. Effect of glycitein and $VEGF_{165}$-induced ERK1/2 activation on RWPE-1 proliferation. (A) Left: RWPE-1 cells were treated with glycitein (50 μmol/L) and U0126 (10 μmol/L) alone and in combination for 72 h, and proliferation was assessed by MTT assay. Right: Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in RWPE-1 cells following treatment with or without U0126 (10 μmol/L, 1 h) prior to glycitein (50 μmol/L, 2 h). (B) Left: RWPE-1 cells were treated with $VEGF_{165}$ (50 μg/L) and U0126 (10 μmol/L) alone and in combination for 72 h, and proliferation was assessed by MTT assay. Right: Active ERK1/2 (p-ERK1/2) was measured by immunoblotting in RWPE-1 cells following treatment with or without U0126 (10 μmol/L, 1 h) prior to $VEGF_{165}$ (50 μg/L, 10 min). Total ERK1/2 (t-ERK1/2) was used as loading control. All immunoblotting and proliferation experiments were performed independently at least thrice, with $n=3$–6 per experiment. Data are presented as percentages of mean±RSE. Mean values not sharing a common superscript letter differ significantly ($P<.05$).

Treatment of RWPE-1 cells with $VEGF_{165}$ (50 μg/L) in combination with U0126 (10 μmol/L) inhibited cellular proliferation by 25%, compared to controls receiving vehicle alone ($P<.01$).

ERK1/2 activation in RWPE-1 cells following treatment with $VEGF_{165}$ (50 μg/L, 10 min) and U0126 (10 μmol/L, 1 h) alone or in combination is presented in Fig. 6B (right panel). $VEGF_{165}$ treatment alone increased ERK1/2 activity, while pretreatment with U0126 inhibited both basal and $VEGF_{165}$-induced ERK1/2 activation.

4. Discussion

Total soy isoflavone concentrations in prostatic fluid can reach up to 50 μmol/L in persons consuming a soy-rich diet [24,25]. Isoflavones in prostatic fluid are in direct contact with the prostate epithelium, suggesting a potential role for these compounds to modulate epithelial cellular function. ERK1/2 signaling cascade is necessary, in part, for the survival, growth and development of the normal prostate epithelium [21]. However, the role of ERK1/2 signaling in prostate carcinogenesis is unclear [21,26–31]. Both increased and decreased levels of ERK1/2 activity have been observed with prostate cancer development. We have previously shown that genistein increases ERK1/2 activity and cellular proliferation at physiological concentrations (<12 μmol/L) in RWPE-1 cells, while higher concentrations (>12.5 μmol/L) decreased ERK1/2 signaling and the proliferation of this cell line [15].

Although genistein is the most abundant isoflavone found in soy, it has relatively low concentrations in prostatic fluid (≤1 μmol/L) [24,25]. The isoflavones daidzein and equol have been shown to selectively accumulate in prostatic fluid to a greater extent than genistein, suggesting a potential for greater biological and/or anticancer activity. The concentration of glycitein in prostatic fluid has not been reported. To date, the anticancer activities of isoflavones other than genistein in the prostate have not been extensively characterized. The present study examined the effects of major and minor soy isoflavones on ERK1/2 signaling and cellular proliferation in nontumorigenic prostate epithelial cells.

Of the isoflavones tested, glycitein-induced ERK1/2 activation in RWPE-1 cells was the most robust. Biological effects of isoflavones are mediated, in part, via the modulation of sex hormone (i.e., estrogen) signaling [15–20]; however, glycitein-induced ERK1/2 activation was independent of estrogen and androgen receptor signaling. The low estrogenic activity of glycitein is attributed, in part, to the 6-methoxy group interfering with the estrogenic functionality of the 7-hydroxyl group on this isoflavone [32].

The activation of receptor and non-receptor tyrosine kinases (RTKs) is an early initiating event in the activation of multiple cellular signaling pathways, including ERK1/2 cascade. Genistein is a known inhibitor of tyrosine kinase activity and has been previously shown to inhibit ERK1/2 via this mechanism. However, the effects of other isoflavones on tyrosine kinase and ERK1/2 activation have not been well characterized. We hypothesized that the effects of glycitein on ERK1/2 activity may involve a tyrosine-kinase-dependent mechanism. To determine the potential involvement of tyrosine kinases in glycitein-induced ERK1/2 activation, several chemical inhibitors of RTK and non-RTK activity were utilized. Of those inhibitors tested, only the VEGFR tyrosine kinase inhibitor blocked glycitein-induced ERK1/2 activation. VEGFRs are predominantly expressed in endothelial cell types; however, more recently, this family of receptors has been characterized in intestinal epithelial cells, keratinocytes [33,34] and a variety of cancer cells [35–42]. The inability of soy isoflavones to activate ERK1/2 in the PC-3 cell line may be due, in part, to the absence of VEGFRs in this cell line [40,42,43].

Activation and signaling via VEGFRs control a host of cellular functions, including proliferation, differentiation and vascular permeability. ERK1/2 activation has been shown to mediate many of these VEGFR-associated cellular effects [44]. Data presented in this study demonstrate the presence of both VEGFR1 and VEGFR2 in the RWPE-1 cell line. In addition, two lines of evidence suggest the functionality of VEGFR in the RWPE-1 cell line: first, the natural ligand for VEGFR, $VEGF_{165}$, significantly increased RWPE-1 proliferation; and, second, $VEGF_{165}$-induced cell proliferation was ERK1/2-dependent (Fig. 6B). Interestingly, glycitein-induced ERK1/2 was associated with decreased RWPE-1 cell proliferation. We hypothesize that the differential effects of $VEGF_{165}$ and glycitein on cellular proliferation may be explained, in part, by the duration of ERK1/2 activity elicited by each of these compounds. Transient activation of ERK1/2 is associated with increased cellular proliferation, whereas sustained activation of this kinase can lead to increased cellular differentiation [45]. The effects of soy isoflavones on sustained ERK1/2 activation and prostate differentiation have not been examined in vivo. However, the accumulation of isoflavones in prostatic fluid following long-term dietary soy consumption [24] suggests a potential role for these compounds in modulating cell signaling, proliferation and differentiation in the prostate.

The present study demonstrates that glycitein induces sustained ERK1/2 activation at concentrations ranging from 0.01 to 100 μmol/L in RWPE-1 cells. This activation was dependent on VEGFR signaling and resulted in decreased cellular proliferation. These biological effects may contribute, in part, to the anticancer activity of soy in the prostate. The influence of soy isoflavones on prostate epithelial differentiation is currently under investigation in this laboratory.

Acknowledgments

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