

Effect of Genistein on p90RSK Phosphorylation and Cell Proliferation in T47D Breast Cancer Cells

JOHN GWIN¹, NEIL DREWS¹, SYED ALI¹, JUSTIN STAMMSCHROR¹,
MATTHEW SORENSON² and TALITHA T. RAJAH¹

Departments of ¹Biological Sciences and ²Nursing, DePaul University, Chicago, IL 60614, U.S.A.

Abstract. *Background: The molecular mechanisms of genistein's proliferative effects on breast cancer cells are largely unknown. This study aimed to examine estrogen-receptor (ER)-related signaling molecules involved in genistein-associated cell proliferation and survival (ERK1/2, p90RSK, JNK, Akt and NFκB) and to correlate these results to cell proliferation. Materials and Methods: The effect of genistein on cell-signaling molecules was determined in T47D breast cancer cells by a Bioplex phosphoprotein detection kit. These results were confirmed by Western blotting and were correlated to cell proliferation by MTT assay. Results: Low and high concentrations of genistein induced an ERK1/2-independent decrease in phosphorylated p90RSK. This effect was accompanied by decreased cell proliferation at high concentrations and an increased response at low concentrations of genistein following a 48-hour exposure. Conclusion: Concentration-dependent actions of genistein in T47D cells may be due to differential activation of signaling molecules.*

The use of complementary or alternative therapies such as soy to alleviate menopausal symptoms and prevent cardiovascular disease risk has gained popularity over the last decade. Genistein, a major component of soy, is able to bind to estrogen receptor (ER) α and/or β and mimic or antagonize the effects of endogenous estrogens (1, 2).

Previous studies have shown that genistein inhibits breast cancer cell growth in a biphasic manner by increasing cell proliferation at low concentrations and decreasing cell proliferation at high concentrations (2-4). However, the molecular mechanism(s) by which genistein induces this biphasic cell proliferative response is unclear.

Correspondence to: Talitha T. Rajah, Ph.D., Department of Biological Sciences, DePaul University, 2325 N. Clifton Ave, Chicago, IL 60614, U.S.A. Tel: +1 7733258006, Fax: +1 7733257596, e-mail: trajah@depaul.edu

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Previous studies have demonstrated that the biphasic effect of genistein on breast cancer cell proliferation depends on the ER status of the cell line (2). Genistein exerts a biphasic cell proliferative response in the presence and absence of 17β-estradiol in T47D breast cancer cells that harbor both ERα and ERβ. However, this biphasic response is not observed in MDA-MB-231 breast cancer cells that only contain ERβ. Sotoca *et al.* showed that the cell proliferative effect of genistein is inversely proportional to the ratio of ERα/ERβ in T47D cells (5). Wild-type T47D cells have a predominance of ERα over ERβ mRNA (9:1) (6). However, genistein-induced cell proliferation is inhibited when ERβ expression is introduced into wild-type T47D cells in a concentration-dependent manner (5). This finding suggests that the effect of genistein on cell proliferation is predominantly mediated via ERα. It is therefore hypothesized that genistein may influence breast cancer cell proliferation through ER-mediated cell signaling pathways that regulate cell growth and survival. There are only few reports on the molecular mechanism underlying the genistein-induced biphasic cell proliferative response in T47D breast cancer cells. The present study sought to examine key ER-mediated signaling molecules involved in genistein-mediated cell proliferation and survival in breast cancer cells.

Estrogen binds to ERs and influences cell growth, differentiation and development of the male and female reproductive tissues. Signaling through ERs occurs via a diverse array of intracellular signaling molecules from several different signal transduction pathways, including the extracellular signal-regulated kinase (ERK1/2), protein kinase B (Akt/PKB) and nuclear factor kappa B (NFκB) pathways (7, 8).

The ERK pathway signaling molecules that are activated through the ER include Ras, B-Raf, and ERK1/2 (9). The roles of ERK1/2 in influencing cell growth, differentiation and development are well established. The downstream molecule activated by ERK1/2 is p90 ribosomal S6 kinase (p90RSK). The p90RSK protein phosphorylates the target substrates that are involved in cell proliferation and survival. c-Jun N-terminal kinase (JNK), a stress response protein that

serves as a mediator of cell death, is activated by estrogen through the ER in human breast cancer cells (10, 11).

Estrogen activates the phosphoinositide 3-kinase (PI3K) pathway to stimulate Akt, both of which regulate the balance between cell survival and apoptosis (12). NF κ B is a key regulator of the inflammatory process, cell proliferation and apoptosis and is widely expressed in breast cancer and normal mammary gland development (13, 14). Activated NF κ B is responsible for inhibiting cell growth and inducing apoptotic cell death. Studies have shown that apoptosis may be induced by estrogen *via* repression of NF κ B (12, 15).

The present study examined the effect of genistein on phosphorylated target proteins involved in cell proliferation and survival, namely ERK1/2, p90RSK, Akt, JNK and NF κ B. Both ERK1/2 and its downstream signaling molecule, p90RSK, regulate cell proliferation. JNK, NF κ B and Akt are involved in cell death and survival. To correlate the signaling events with cellular behavior, the effect of genistein on breast cancer cell (T47D) proliferation was determined at 24 and 48 hours. Given that human exposure to soy has increased, elucidating the pleiotropic nature of phytoestrogens in altering ER-mediated signaling networks is highly important, especially for women with ER-positive breast cancer.

Materials and Methods

Cell culture. T47D cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown as a monolayer in phenol red-free RPMI-1640 supplemented with 2 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.2 U/ml bovine insulin, 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μ g/ml) (all from Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in 75 cm² culture flasks at 37°C with 5% CO₂ and 95% humidity and were passaged once a week by routine trypsinization.

Treatment conditions. Genistein was obtained from Sigma-Aldrich. Genistein was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and added to the cell cultures following dilution in serum-free culture medium. The DMSO vehicle was used at a final concentration of 0.1% in growth medium and had no effect on cell proliferation. The control cultures received 0.1% of the vehicle alone.

Cell proliferation. The effect of genistein on cell proliferation was assessed using the MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay, as described previously (2). The concentrations of genistein were chosen from a previous study (2) and included the low concentration range (1 nM to 10 μ M) and the high concentration range (25 μ M to 100 μ M). The MTT assay is commonly used to assess cell proliferation and viability by measuring the reduction of yellow MTT by mitochondrial dehydrogenases in viable cells. This yields purple formazan crystals that may be detected colorimetrically at 590 nm. The MTT assay was performed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, breast cancer cells were seeded in 24-well plates at approximately 3 \times 10⁵ cells/well. Cells were allowed to attach overnight and, then, were estrogen-starved for 24 hours by replacing

the medium with phenol red-free RPMI-1640 containing a B-27 supplement (Invitrogen, Grand Island, NY, USA). B-27 supplements are completely devoid of estrogen and serve as serum-free supplements for cell growth and viability. Following estrogen starvation, the cells were exposed to various concentrations of genistein (1 nM to 100 μ M) for 24 or 48 hours. Cells were then incubated for two hours in the presence of MTT at 10% of the cell culture volume. The resulting formazan crystals were dissolved in MTT solubilization solution (10% Triton X-100, 0.1 N HCl in isopropanol). The absorbance was measured at 570 nm and the background absorbance was measured at 690 nm. The background reading was subtracted from the absorbance measurement and the cell survival was calculated as a percentage of the control.

Bioplex phosphoprotein assay. Expression of phosphorylated proteins of pERK1/2 (Thr²⁰²/Tyr²⁰⁴, Thr¹⁸⁵/Tyr¹⁸⁷), p90RSK (Thr³⁵⁹/Ser³⁶³), Akt (Ser⁴⁷³), JNK (Thr¹⁸³/Tyr¹⁸⁵) and NF κ B (Ser⁵³⁶) were detected by the commercially available Bioplex phosphoprotein assay kit (Bio-Rad, Hercules, CA, USA), as described previously (16). Briefly, cells at 50% confluence were estrogen-starved for 24 hours and treated with genistein at 1 μ M and 100 μ M for 30 minutes. Protein lysates were collected by using a whole-cell protein extraction kit (Chemicon International, Temecula, CA, USA). Protein concentrations were estimated by the Bradford Assay (Bio-Rad) and adjusted to a concentration of 100-500 μ g/ml. Protein lysates were plated in the 96-well filter plate coated with beads coupled to anti-phosphorylated target proteins (ERK1/2, p90RSK, Akt, JNK and NF κ B) and were incubated overnight at room temperature on a platform shaker at 300 rpm. After a series of washes to remove unbound proteins, a mixture of biotinylated detection antibodies, each specific for a different epitope, was added. Streptavidin-phycoerythrin was subsequently added to bind the biotinylated detection antibodies on the bead surface. The data from the reaction were collected and analyzed using the Bioplex suspension array system. Each phosphorylated protein was normalized to the total myosin protein.

Western blot analysis. Breast cancer cells were seeded into 100-mm tissue culture dishes, allowed to reach 60-70% confluence and estrogen-starved for at least 24 hours. The cells were then treated with genistein (1 or 100 μ M) for 30 minutes. Control cells were treated with the solvent DMSO (0.1%). Proteins were extracted with the whole-cell extraction kit according to the manufacturer's protocol and quantified by the Bradford assay. Approximately 50 μ g of total protein was loaded onto a precast 10% acrylamide gel (Thermo Fisher Scientific, Rockford, IL, USA) and transferred to a nitrocellulose membrane. Blots were incubated in Tris buffered saline with 0.1% Tween 20 (TBS-T) plus 5% nonfat dry milk for one hour at room temperature and rinsed three times in TBS-T. Blots were then incubated with primary antibodies against p90RSK (Total RSK1/2/3) and phosphorylated p90RSK (Thr³⁵⁹/Ser³⁶³; Cell Signaling Technology, Beverly, MA, USA) in TBS-T plus 5% BSA overnight at 4°C. Following primary antibody incubation, blots were washed three times with TBS-T, incubated for one hour at room temperature with horseradish peroxidase-conjugated secondary antibodies and washed three times in TBS-T. Protein bands were visualized using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Chemiluminescent images were captured by a Chemo-Imager (Alpha Innotech, Santa Clara, CA, USA) and optical density was quantified with the AlphaView software, version 3.0 (Alpha Innotech, Santa Clara, CA, USA).

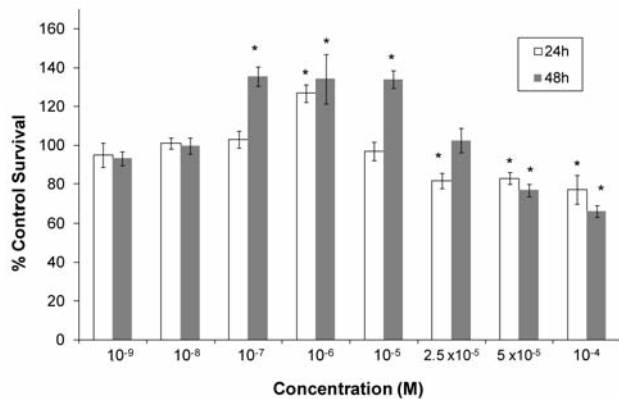


Figure 1. Effect of genistein on T47D human breast cancer cell proliferation. Cells were seeded in 24-well plates and allowed to attach overnight. Following estrogen starvation, cells were treated with genistein (1 nM to 100 μ M) for 24 and 48 hours. Cell survival was determined by the MTT assay and calculated as percentage of the vehicle-treated control. Data are presented as mean \pm standard error of the mean from at least three different experiments; * $p < 0.05$ as compared to the control.

Expression of phosphorylated p90RSK in optical density units was normalized to total p90RSK and expression of phosphorylated p90RSK was expressed in relative density units.

Data analysis. Data are presented as mean \pm standard error of the mean of at least three independent experiments. The data were analyzed for statistical significance using the Student's *t*-test (Microsoft Excel 2007) and $p < 0.05$ was considered statistically significant.

Results

T47D cells exposed to genistein for 48 hours showed a biphasic cell proliferative response in the presence of physiological concentration of 17 β -estradiol. Low concentrations of genistein (100 nM, 1 μ M and 10 μ M) significantly stimulated cell growth at 48 hours (about 140%; $p < 0.05$ for each) as determined by the MTT assay (Figure 1). However, after only 24 hours of low-concentration genistein exposures a significant increase in cell growth was observed only at 1 μ M genistein (approximately 135%; $p < 0.05$). High concentrations of genistein (25 μ M to 100 μ M) induced a significant 20-40% decrease in cell proliferation at 24 and 48 hours as compared to the control ($p < 0.05$) (Figure 1). These results suggest that the threshold concentration for the biphasic response by genistein lies between 10 μ M and 25 μ M.

Figure 2 shows the phosphorylation status of five signaling molecules (Akt, ERK1/2, JNK, p90RSK and NF κ B) involved in cell proliferation, survival and death following genistein treatment (1 μ M or 100 μ M) in T47D cells. Phosphorylation was determined by the Bioplex phosphoprotein assay. Based

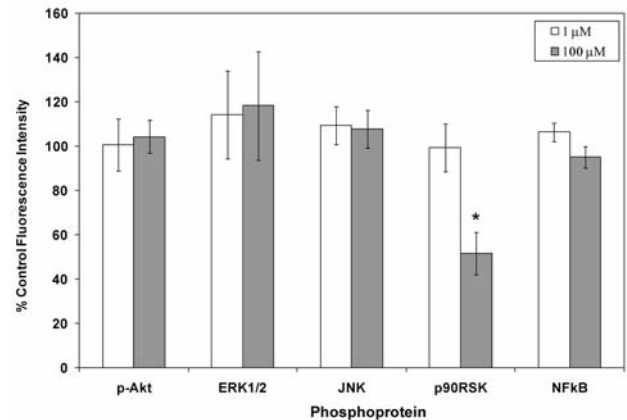


Figure 2. Effects of genistein on different phosphorylated proteins in T47D human breast cancer cells. Cells at approximately 50% confluence were estrogen-starved for 24 hours and treated with genistein at 1 μ M or 100 μ M for 30 minutes. Total proteins were extracted, and the protein concentration was adjusted to 100-500 μ g/ml. The presence of phosphorylated pERK1/2, p90RSK, Akt, JNK and NF κ B was detected by the Bioplex phosphoprotein assay kit. Data are presented as mean \pm standard error of the mean from at least three different experiments; * $p < 0.05$ as compared to the control.

on the results from the cell proliferation studies, 1 μ M genistein was chosen to represent a low concentration and 100 μ M a high concentration. A 50% decrease in phosphorylated p90RSK occurred following 100 μ M genistein exposure ($p < 0.05$) as compared to the control. The other phosphorylated proteins studied did not show appreciable differences at either concentration of genistein.

The significant decrease in phosphorylated p90RSK that was observed using the Bioplex phosphoprotein assay was confirmed by Western blotting. There was a significant decrease ($p < 0.05$) in phosphorylated p90RSK (approximately 40%) in T47D cells exposed to 100 μ M genistein as compared to the control (Figure 3). The p90RSK phosphorylation results obtained by Western blotting validate the results produced by the Bioplex phosphoprotein assay that revealed a 50% inhibition of p90RSK phosphorylation by 100 μ M genistein. Using Western blot analysis, a decrease in phosphorylated p90RSK was also seen in cells exposed to 1 μ M genistein, but this difference was not statistically significant. The decrease in phosphorylated p90RSK at 1 μ M genistein was not observed with the Bioplex phosphoprotein assay and revealed the sensitivity of the Western blot analysis over the Bioplex phosphoprotein assay for this protein.

Discussion

The objective of this study was to determine the molecular mechanism underlying concentration-dependent effects of genistein on cell proliferation in T47D breast cancer cells. The

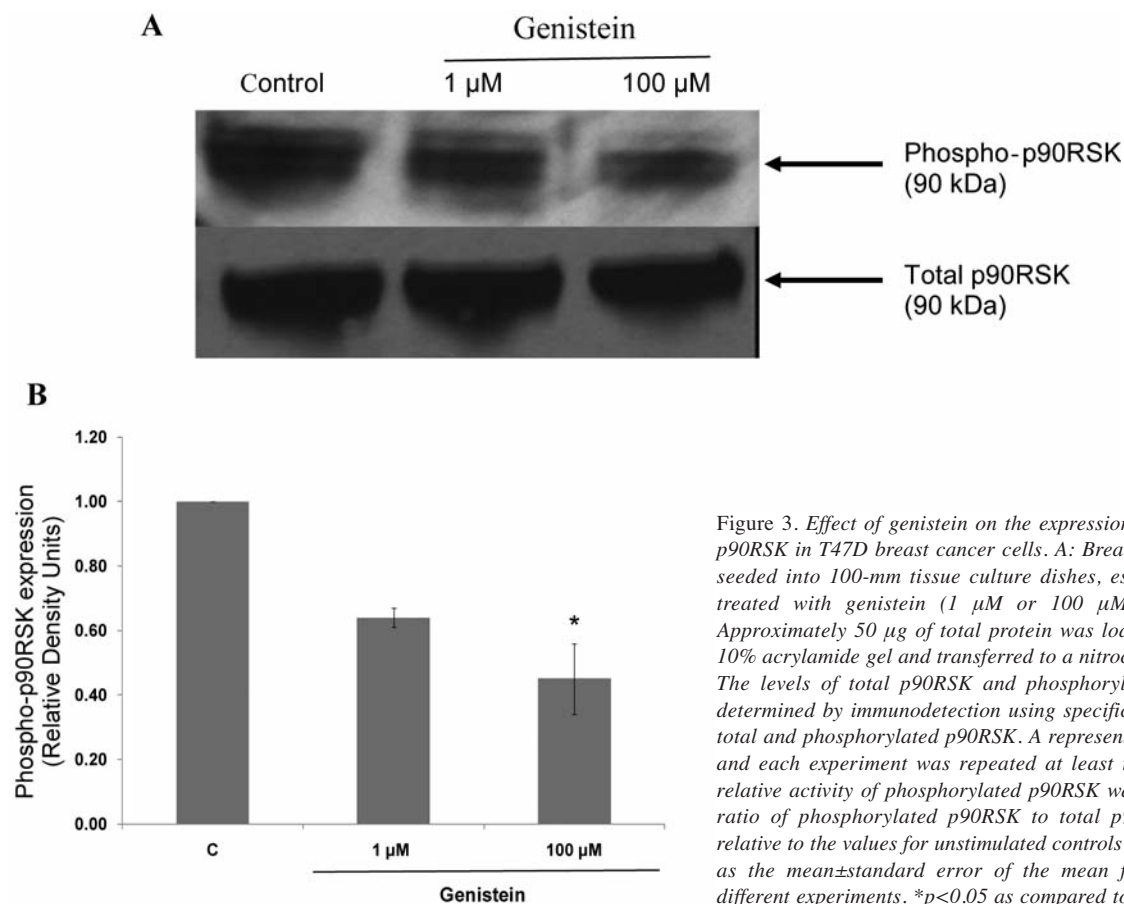


Figure 3. Effect of genistein on the expression of phosphorylated p90RSK in T47D breast cancer cells. A: Breast cancer cells were seeded into 100-mm tissue culture dishes, estrogen-starved and treated with genistein (1 μ M or 100 μ M) for 30 minutes. Approximately 50 μ g of total protein was loaded onto a precast 10% acrylamide gel and transferred to a nitrocellulose membrane. The levels of total p90RSK and phosphorylated p90RSK were determined by immunodetection using specific antibodies against total and phosphorylated p90RSK. A representative blot is shown, and each experiment was repeated at least three times. B: The relative activity of phosphorylated p90RSK was calculated as the ratio of phosphorylated p90RSK to total p90RSK and plotted relative to the values for unstimulated controls. Data are presented as the mean \pm standard error of the mean from at least three different experiments. * $p < 0.05$ as compared to the control.

study evaluated the expression of phosphorylated signaling molecules involved in cell proliferation and survival (ERK1/2, p90RSK, JNK, Akt and NF κ B) in T47D cells treated with genistein (1 μ M and 100 μ M). The study revealed that p90RSK phosphorylation is significantly inhibited by 40-50% by 100 μ M genistein as compared to the control.

This is the first report to show that high concentrations of genistein significantly inhibit the phosphorylation of p90RSK. A recent study showed that equol, a metabolite of the soy isoflavone daidzein, inhibits the activation of p90RSK and decreases neoplastic transformation in JB6 P+ mouse epidermal cells (17). The signaling molecule p90RSK is downstream of ERK1/2 in the MAPK pathway and regulates multiple signaling molecules involved in cell proliferation and survival (18). p90RSK has two functional kinase domains, a C-terminal domain that is involved in the activation of p90RSK and an N-terminal domain that activates the substrates of p90RSK. Both ERK1/2 and phosphoinositide-dependent kinase (PDK) 1 are required for the activation of p90RSK. The p90RSK C-terminal kinase domain is activated by ERK1/2, which also phosphorylates the linker region between the C- and N-terminus. The phosphorylated linker region serves as a docking

site for PDK1, which in turn phosphorylates the N-terminus of p90RSK to induce p90RSK activation. The downstream targets of phosphorylated p90RSK are transcription factors from multiple signaling pathways of cell proliferation and survival, including ER α and BAD (18). The phosphorylated p90RSK antibody (anti-P-p90RSK Thr³⁵⁹/Ser³⁶³) used in this study detects ERK1/2-induced phosphorylation of p90RSK in the linker region at Thr³⁵⁹ and Ser³⁶³ (18, 19).

The results of the present study show that phosphorylation of p90RSK is inhibited by high concentrations of genistein even though the upstream signaling molecule ERK1/2 is not inhibited. Indeed, the effect of genistein on ERK1/2 phosphorylation appears to be concentration- and cell-type-dependent (20). ERK1/2 shows delayed activation by genistein (10 μ M) in MCF-7 cells (21) but is stably activated by genistein (20 μ M) in MDA-MB-231 cells (22). According to other studies, ERK1/2 is inhibited by genistein (50 μ M) in T47D cells (20) and in CaSki cervical cancer cells (20 and 60 μ M) (23). However, p90RSK, the downstream target of ERK1/2, was not evaluated in those studies. In studies that examined both ERK1/2 and p90RSK, these molecules appear to be coordinately activated and inactivated (18).

It is difficult to interpret the present results showing that phosphorylation of p90RSK is inhibited by a high concentration of genistein without a concomitant inhibition of ERK1/2 phosphorylation. These results suggest that high concentrations of genistein may activate p90RSK in T47D breast cancer cells *via* signaling molecules other than ERK1/2. Several studies have shown that reactive oxygen species (ROS) alter the phosphorylation status of p90RSK in an ERK1/2-independent manner (24, 25). ROS-induced stimulation of phosphorylated p90RSK occurs in the absence of a coordinated increase in phosphorylated ERK1/2 in Jurkat cells (24). Furthermore, free-radical scavengers such as vitamin E and *N*-acetyl cysteine abolish the ROS-mediated stimulation of p90RSK phosphorylation and growth stimulation of prostate tumor spheroids in the absence of activated ERK1/2 inhibition (25).

Genistein is a well-established and concentration-dependent antioxidant with free-radical scavenging abilities (26). It is possible that inhibition of phosphorylated p90RSK occurs in an ERK1/2-independent manner due to the antioxidant properties of genistein. Cancer cells have high levels of ROS, including H₂O₂ and reduced levels of free-radical scavenging enzymes, such as catalase (27). Taken together, these results suggest that ERK1/2-independent inhibition of phosphorylated p90RSK may occur through the ROS signaling pathway rather than the Ras-mediated MAPK pathway, at least at high concentrations of genistein in T47D cells. The lack of coordinated regulation between ERK1/2 and p90RSK also suggests that additional ERK1/2 phosphorylation time points may need to be examined. However, further studies are required to delineate the signaling molecules upstream of p90RSK that mediate genistein actions in T47D cells.

Inhibition of p90RSK may lead to decreased cell growth in T47D breast cancer cells *via* its activity on different substrates involved in cell proliferation and survival (ER α and BAD). ER α is phosphorylated by p90RSK at Ser 167, which leads to the activation of ER in the absence of a ligand (28). It is possible that inhibition of p90RSK in T47D cells may result in decreased ER phosphorylation and suppression of cell growth.

Western blot analysis revealed that low concentrations of genistein inhibited p90RSK phosphorylation, but these reductions were not significant. These results suggest that stimulation of T47D cell proliferation by low concentrations of genistein may be due to cell-proliferative signaling molecules that were not examined in this study.

There were no significant differences observed in the expression of Akt, NF κ B or JNK following genistein treatment in T47D cells. A previous study in T47D cells showed that a low concentration (1 μ M) of genistein increases Akt phosphorylation, but a higher concentration (100 μ M) has no effect (29). Genistein inhibits NF κ B induction after treatment with H₂O₂ and tumor necrosis

factor- α in prostate cancer cells and human blood lymphocytes (26, 30). The differences between the results of the present and earlier studies may be due to cell line differences and varying genistein treatment times.

To correlate the signaling events with cellular behavioral responses, the effect of genistein on cell proliferation was studied at 24 and 48 hours, based on the assumption that these would be the earliest time points for the manifestation of a behavioral response to the signaling events. T47D cells that contained both ERs showed a biphasic proliferative response following genistein treatment for 48 hours. Proliferation was stimulated at low concentrations and inhibited at high concentrations (Figure 1). Similar results with genistein have been observed in T47D cells when the cells were cultured for 72 hours, but the responses were more pronounced (2). Previous studies with other cells that contain both ERs, such as MCF-7 breast cancer cells, have shown similar biphasic cell-proliferative responses (2-4). A previous study showed that such a biphasic response occurs in cells that contain both ERs but not in cells that contain only ER β (2). This suggests that the stimulation of cell proliferation that is seen at low concentrations of genistein is possibly mediated by ER α . This is further supported by the fact that wild-type T47D breast cancer cells have a 9:1 ratio of ER α : ER β mRNA (6). A recent study using T47D cells containing tetracycline-dependent ER β expression showed that the growth-stimulatory effects of low concentrations of genistein are inhibited when the expression of ER β is increased (5). Taken together, these results suggest that even though p90RSK phosphorylation was inhibited by low concentrations of genistein, other ER α -related cell-proliferative signaling molecules that were not included in this study may be involved in the genistein-induced stimulation of cell proliferation.

A statistically significant decrease in T47D cell proliferation occurred after treatment with high concentrations of genistein (25 μ M to 100 μ M). It has been postulated that genistein acts as a cytotoxic agent by inducing apoptosis at high concentrations (31). This study showed that phosphorylated p90RSK, a cell-proliferative signaling molecule, is significantly inhibited in an ERK1/2-independent manner following treatment with high concentrations of genistein.

In conclusion, the results of the present study suggest that high concentrations of genistein alter T47D cell proliferation by inhibiting phosphorylation of p90RSK in an ERK1/2-independent manner. The mechanistic effects of genistein are concentration-dependent and different signaling molecules are activated at different concentrations.

Acknowledgements

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