Molecular effects of genistein on estrogen receptor mediated pathways

Thomas T.Y. Wang, Neerja Sathyamoorthy and James M. Phang

Laboratory of Nutritional and Molecular Regulation, NCI—Frederick Cancer Research and Development Center, NIH, Frederick, MD 21702-1201, USA

Genistein, a component of soy products, may play a role in the prevention of breast and prostate cancer. However, little is known about the molecular mechanisms involved. In the present study, we examined the effects of genistein on the estrogen receptor positive human breast cancer cell line MCF-7. We observed that genistein stimulated estrogen-responsive pS2 mRNA expression at concentrations as low as $10^{-8}$ M and these effects can be inhibited by tamoxifen. We also showed that genistein competed with [3H]estradiol binding to the estrogen receptor with 50% inhibition at $5 \times 10^{-7}$ M. Thus, the estrogenic effect of genistein would appear to be a result of an interaction with the estrogen receptor. The effect of genistein on growth of MCF-7 cells was also examined. Genistein produced a concentration-dependent effect on the growth of MCF-7 cells. At lower concentrations ($10^{-8}$–$10^{-6}$ M) genistein stimulated growth, but at higher concentrations ($>10^{-5}$ M) genistein inhibited growth. The effects of genistein on growth at lower concentrations appeared to be via the estrogen receptor pathway, while the effects at higher concentrations were independent of the estrogen receptor. We also found that genistein, though estrogenic, can interfere with the effects of estradiol. In addition, prolonged exposure to genistein resulted in a decrease in estrogen receptor mRNA level as well as a decreased response to stimulation by estradiol.

Introduction

In epidemiological studies, an increased intake of soy products is associated with a decreased risk of hormone-dependent cancers (1, 2). In rodents, ingestion of soy products was found to inhibit mammary tumorigenesis (3). It has been suggested that genistein, an isoflavone abundant in soy products, may contribute to the chemopreventive effects of soy products for hormone-dependent cancers (2, 4). However, the molecular mechanisms by which genistein exerts its effects remain poorly understood. This is not due to a lack of proposed and identified effects. On the one hand, genistein has been shown to stimulate proliferation, presumably through estrogen-like effects (5). On the other hand, it is considered to be antiproliferative, presumably due to inhibition of tyrosine kinases (6–8) and topoisomerase (9). Until its mechanisms of action are better understood, the chemopreventive potential of genistein is unlikely to be fully realized.

In the present study, we focused on the effect of genistein on the estrogen receptor (ER*) pathway. Using the human breast cancer MCF-7 cells, we examined the effects of RNA on: (i) the expression of pS2 mRNA; (ii) the displacement of estrogen from binding to the ER; (iii) cellular proliferation; and (iv) regulation of the ER. The results from these studies were used to address the following questions. (i) Is genistein estrogenic? (ii) Could genistein be anti-estrogenic? (iii) What contributes to the paradoxical observation that genistein stimulates both proliferative and anti-proliferative responses?

Materials and methods

Chemicals

Genistein was purchased from Sigma Chemical Co. (St Louis, MO). Quercetin was purchased from Indofine Chemical Co. (Somerville, NJ). Tamoxifen was purchased from Aldrich Chemical Co. (Milwaukee, WI). These compounds were >95% pure. All other chemicals were from the best sources available.

Cells and cell culture

MCF-7 cells were maintained in Medium A (RPMI 1640 with 2 mM L-glutamine, hydrocortisone (3.5 ng/ml), insulin (1.5 ng/ml), penicillin (100 U/ml) and streptomycin (100 mg/ml) with 5% fetal bovine serum in 75 cm\(^2\) flasks (Falcon) and grown in the presence of 5% CO\(_2\) in air at 37°C. One week before initiation of the experiment, cells were switched to Medium A supplemented with 5% charcoal dextran-treated fetal bovine serum (FCS). Cells were grown to confluence and passaged using trypsin-EDTA. Viable cells were counted by Trypan blue exclusion. A day prior to treatment the cells were switched to Medium B [phenol-red free RPMI 1640 containing 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% FBS].

Probe for pS2 and ER

digoxigenin (DIG)-labeled pS2 cRNA probe (10) was synthesized using a nucleic acid labeling kit following the manufacturer’s instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). For production of the DIG-ER cRNA probe, plasmid pOR3 carrying the ER cDNA (11) was obtained from the American Type Culture Collection. The pOR3 was digested with EcoRI and a 1.35 kb cDNA fragment coding for the ER was inserted into the EcoRI site of plasmid pGEM-4Z. The resulting plasmid was designated pGEM/ER. DIG-labeled ER cRNA probe was generated from NarI linearized pGEM/ER using the SP6 RNA polymerase following the procedure described previously (10).

Total RNA isolation, Northern blot hybridization and detection

For isolation of total RNA, MCF-7 cells were grown in six-well Costar plates (1 \times 10^{6} cells/well in 3 ml of Medium B) and isolated as described previously (10). RNA was resuspended in 70–100 µl DEPC-treated water, and 10 µl of RNA was routinely used for Northern blot analysis. For detection of mRNA, 10 µl of total RNA was separated on a 1% agarose formaldehyde gel and transferred to nylon membrane (Boehringer Mannheim) using the PossiBlot Pressure Blotter (Stratagene, La Jolla, CA). The RNA was fixed onto the membrane by UV crosslinking in a Stratalinker (Stratagene) for 3 min at the highest power. The membrane was then hybridized with DIG-labeled RNA probe and detected by a chemiluminescence nucleic acid detection method according to the manufacturer’s protocol (Boehringer Mannheim). All RNA samples were also probed for either glyceraldehyde 3-phosphate dehydrogenase (G3PDH) or β-actin mRNA, both of which were used to normalize specific expression stimulated by various treatments. After exposure to X-ray film (Kodak, Rochester, NY), the intensity of the pS2, ER, G3PDH or β-actin RNA content and expressed as relative expression level.

Growth assay

Cell growth was determined by the sulforhodamine colorimetric assay as described by Skehan et al. (12) except that the cells were seeded in 24-well
plates instead of 96-microtiter plates. Routinely, 1 x 10^6 cells in 1 ml of Medium B were seeded to each well of a 24-well plate (Costar, Cambridge, MA). Twenty-four hours after plating, either genistein or vehicle was added. The medium was changed every 24 h and the test compounds were replenished with each medium change. After color development, aliquots were pipetted into a 96-well microtiter plate and the intensity of the developed color was measured at 570 nm using an ELISA microplate reader (Molecular Devices, Menlo Park, CA).

ER binding study

MCF-7 cells were grown to confluence in 150 cm^2 flasks (Becton Dickinson Labware, Lincoln Park, NJ) and cultured in Medium A supplemented with 5% fetal bovine serum at 37°C in the presence of 5% CO_2 in air. The cells were rinsed twice with PBS, scraped off in 5 ml of PBS and pelleted at 1500 rpm for 10 min. After aspiration of the supernatant, the cell pellets were suspended in lysis buffer consisting of 10 mM Tris–HCl pH 7.4, 1.5 mM EDTA and 0.5 mM dithiothreitol. The cell suspension was homogenized using a Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) for 45 s. The homogenate was then centrifuged at 40 000 rpm for 50 min and the supernatant saved. After determination of the protein concentration, the supernatant was used for ER binding assays. ER binding assay was performed essentially as described by McGuire (13). Briefly, the ER binding studies were carried out as follows: 200 μl of the cytosol fraction (2.0 mg/ml protein) was incubated with 10^{-9} M [^3H]estradiol (New England Nuclear, Boston, MA) for 18 h at 4°C. Following incubation, 0.5 ml of dextran-coated charcoal was added to the assay mixture, and incubated at 4°C for 30 min with vortexing at 5 min intervals. The assay mixture was then centrifuged at 2000 g for 10 min and 0.5 ml of the supernatant was removed for measurement of the bound [^3H]estradiol by liquid scintillation spectrometry. For competition experiments, binding assays were run as described above in the presence of increasing amounts of competitor (0–10^{-6} M) while the concentration of estradiol was held constant at 10^{-9} M.

Results

Effect of genistein on pS2 mRNA expression and estradiol binding to ER

The structure of the compounds used in the study is illustrated in Figure 1. To characterize the estrogen-like property of genistein we monitored the expression of estrogen-dependent pS2 mRNA using a chemiluminescence detection method reported previously (10). As illustrated in Figure 2, stimulation of pS2 mRNA expression occurred in a concentration-dependent fashion. The stimulatory effect of genistein on pS2 mRNA expression was observed at concentrations as low as 10^{-8} M. As illustrated in Figure 3(A), the stimulation of pS2 expression by genistein (10^{-7} M) could be blocked by the addition of tamoxifen (10^{-5} M). In addition, actinomycin D at a concentration of 3 μg/ml inhibited the genistein-stimulated pS2 expression (Figure 3B). These results support the hypothesis that genistein acts through an ER-mediated transcriptional event.

To characterize further the interaction between genistein and the ER pathway, we examined the ability of genistein to compete with [^3H]estradiol (10^{-9} M) for binding to the ER. As shown in Figure 4, genistein can compete with estradiol for binding to the ER. In contrast, quercetin, which does not stimulate pS2 expression (10), did not compete with estradiol for binding to ER. The concentration required for genistein to produce a 50% inhibition of binding was 5 x 10^{-7} M. These results, together with the results from the pS2 expression assay, supported the interpretation that genistein induces an estrogenic response by interacting with the ER.

Anti-estrogenic effects of genistein

It is known that anti-estrogens such as tamoxifen can also act as weak estrogens (14). Hence, like tamoxifen, genistein may...
Results (mean±SE, n = 3) are expressed relative to control (mean±SE, n= 3) are expressed as a percentage of control.

Effect of genistein on the growth of MCF-7 cells
In addition to a specific end point mediated through interaction with the ER, the effects of genistein on cellular proliferation was also examined. We monitored the growth of MCF-7 cells in response to genistein at concentrations ranging from $10^{-9}$ to $10^{-4}$ M and found that the effect on proliferation of MCF-7 cells was biphasic. Genistein concentration between $10^{-8}$ and $10^{-5}$ M stimulated the growth of MCF-7 cells (Figure 8). However, at concentrations $>10^{-5}$ M, genistein appeared to inhibit proliferation (Figure 8). Since the threshold for the stimulation of proliferation was in the range of concentration for stimulating expression of pS2, we considered that the stimulation of growth was mediated through the ER. Evidence supporting involvement of the ER was found in studies using the ER-negative MDA-MB-231 cells in which genistein did not stimulate growth. However, in these ER-negative cells, the inhibitory effects of genistein ($10^{-7}$–$10^{-4}$ M) remained (Figure 8).

Discussion
The present study focused on genistein’s mechanism of action and on the apparent contradictions in previously published
Results from the present study provide evidence for the anti-estrogenic effects of genistein. We observed that not only does genistein compete with estradiol for binding to the ER, but it also interferes with the effects of estrogen. When genistein and estradiol were added together in the pS2 expression assay, the expression of pS2 mRNA was lower than when these compounds were added individually. In addition, the duration of exposure to genistein also appeared to be important. MCF-7 cells treated with genistein showed a decrease in the response to estrogen as well as a decrease in ER mRNA levels. This is similar to cells treated with estradiol, as has been described by others (17). Taken together, the results support the idea that genistein at physiologically achievable concentrations may, in fact, interfere with the action of estrogen through direct competition for binding to ER and by reducing ER expression. These effects could be considered anti-estrogenic, consistent with the proposed cancer-preventive effects of soy diet.

In summary, our findings provide support for a model in which phyto-estrogens such as genistein, at physiological concentrations, may exert their effect by modulating estrogenic pathways. This interaction may occur directly through competition with estrogen for binding to ER. In addition, prolonged exposure may result in the reduction of ER expression and hence lead to decreased responsiveness to endogenous estrogens.

References

Molecular effects of genistein on estrogen receptor mediated pathways


*Received on May 18, 1995; revised on October 27, 1995; accepted on October 30, 1995*