Estrogen Activities and the Cellular Effects of Natural Progesterone from Wild Yam Extract in MCF-7 Human Breast Cancer Cells

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Abstract: We studied the estrogenic activity and cellular effect of wild yam extract in MCF-7 human breast cancer cells. The extract increased the activity of the progesterone receptor and pS2 genes at the mRNA levels in human breast cancer MCF-7 cells, although the effects were not as prominent as those of 17β-estradiol (E2). Western blot analysis showed that the level of estrogen receptor α protein was down-regulated after treatment with E2 or wild yam extract. Wild yam extract also inhibited proliferation of MCF-7 cells. These data indicate that wild yam extract acts as a weak phytoestrogen and protects against proliferation in human breast carcinoma MCF-7 cells.

Keywords: Menopause; Phytoestrogen; Wild Yam.

Introduction

Women are often concerned about menopausal and postmenopausal symptoms. Menopausal symptoms include vasomotor symptoms, including hot flashes and night sweats; central nervous system (CNS)-related symptoms such as insomnia and changes in memory, concentration, and mood; urogenital symptoms including vaginal dryness, urinary tract infections, and

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urinary urgency; and long-term disease processes including osteoporosis (Barber et al., 2005). Estrogen or combined estrogen and progestin therapy (hormone replacement therapy) has been the cornerstone of the treatment of menopausal symptoms (Barber et al., 2005). Hormone replacement therapy is effective against various symptoms. However, classical hormone replacement therapy has been questioned following various prospective and retrospective clinical studies, which demonstrated increased risks of cardiovascular disease and breast cancer during and after menopause (Dennerstein et al., 2000; Hulley et al., 1998). These concerns have heightened public interest in alternative medicines as a substitute for pharmacological hormone replacement therapy.

The female body fails to produce enough progesterone between ovulation and menstruation and during menopause (http://www.health-science.com/natpro.html). Progesterone is a precursor to many other steroid hormones. Since it is a modulator, its use can greatly enhance overall hormonal imbalance (Lee, 1996). Synthetic progestin as a replacement hormone can cause harmful side-effects such as partial loss of vision, birth defects, and breast cancer (http://www.oasisserene.com/hormones/). Natural progesterone is often promoted as an alternative to synthetic progestin for 2 reasons. First, it appears to have fewer side-effects in many women (Lee, 1996). Second, it may have more benefits for lipid profile enhancement (The Writing Group for the PEPI Trial, 1995), osteoporosis prevention (Prior, 1990), and the treatment of menopausal symptoms (Hargrove et al., 1989), premenstrual syndrome (PMS) (Maxson, 1987; Dalton, 1984), and endometriosis (Vercellini et al., 1996). Furthermore, progesterone prevents or decreases the occurrence of carcinogen- and estrogen-induced mammary tumors. Wild yam extract, known as “natural progesterone (NP)” is popular for alleviation of menopausal symptoms, contains steroidal saponins and sapogenins, including diosgenin (Komesaroff et al., 2001). Here, we examined whether NP, in the form of wild yam extract, has any effects on cell growth and endogenous estrogen receptors in breast cancer MCF-7 cells.

**Materials and Methods**

**Reagents**

17β-estradiol (E2) was purchased from Sigma (St. Louis, MO). ICI 182,780 (ICI) was obtained from ZENECA Pharmaceuticals (Tocris, UK). E2 was dissolved in 100% ethanol. Wild yam extract was purchased from Jiahe-Phytochem (www.jiaherb.com) and provided by Dr. Woongshick Ahn at Catholic University of Korea College of Medicine. All the compounds were added to the medium such that the total ethanol concentration was never higher than 0.15%. An untreated group served as a control.

**Cell Cultures**

ER-positive human breast adenocarcinoma, MCF-7 cells were purchased from ATCC. MCF-7 cells were maintained in phenol red-free RPMI-1640 containing 1x antibiotic/antimycotic mix (Invitrogen, Gaithersburg, MD), 5 mM N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid, and 0.37% sodium bicarbonate, supplemented with 10% fetal
bovine serum (FBS) (HyClone Laboratories, Inc.). Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO₂ and fed every 2–3 days. Ten nM E₂ was used to maximize the response unless otherwise noted.

**RNA Extraction and Reverse Transcription-Polymerase Chain (RT-PCR) Reaction**

MCF-7 cells were grown in 6-well plates in phenol red-free RPMI-1640 containing 5% CD-FBS. Near confluent monolayers were treated with the compounds for 24 hours. The wells were rinsed in PBS and total RNA was isolated by lysing the cells in guanidinium isothiocyanate by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. After extraction, RNA was precipitated by recommended procedures and dissolved in diethylpyrocarbonate-treated water. To synthesize first strand cDNA, 5 µl total RNA was incubated in 0.5 µg of oligo(dT)₁₈ primer (Invitrogen) and 5 µl deionized water at 70°C for 5 min. Reverse transcription reactions were performed using 40 units of M-MuLV reverse transcriptase (Promega Corp.) in 5× reaction buffer (250 mM Tris-HCl; pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM DTT) and 20 mM dNTP mixtures at 37°C for 60 min. The reaction was terminated by heating at 70°C for 10 min, followed by cooling at 4°C. The resulting cDNA was added to the PCR reaction mixture containing 10× PCR buffer (100 mM Tris-HCl; pH 8.3, 500 mM KCl, 15 mM MgCl₂), 25 units of Taq polymerase (TaKaRa, Japan), 4 µl of 2.5 mM dNTP mixtures, and 10 pmole of primers of each. The final volume was 50 µl. The primers used were: human progesterone receptor (PR) sense primer, 5’-CCA TGT GGC AGA TCC CAC AGG AGT T-3’, PR antisense primer, 5’-TGG AAA TTC AAC ACT TAT ATG TGT CCA GCC-3’ (Mercier et al., 2001); ERα sense primer, 5’-CAT AAC GAC TAT ATG TGT CCA GCC-3’, ERα antisense primer, 5’-AAC CGA GAT GAT GTA GCC AGC AGC-3’; pS2 sense primer, 5’-CAT GGA GAA CAA GGT GAT GTG CTA-3’, pS2 antisense primer, 5’-CAG AAG CTT GTC TGA GTG GTC-3’ (Liu et al., 2001); β-actin sense primer, 5’-CCT GAC CCT GAA GTA CCC CA-3’, β-actin antisense primer, 5’-CGT CAT GCA GGT CAT AGC TC-3 (Ren et al., 1997). The PCR-product for PR is 271 bp and 365 bp for pS2 and 550 bp for β-actin. The reactions were initiated by 3 min of denaturation at 94°C followed by amplification at 94°C for 45 sec, and 55°C for 45 sec, and 72°C for 45 sec; 24 cycles for PR or pS2 and 20 cycles for β-actin. The PCR reaction was ended by elongation at 72°C for 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining, quantified by using a bio-imaging analyzer (Bio-Rad Laboratories, Inc., Hercules, CA), and band-intensity was normalized to the intensity of β-actin mRNA product.

**Western Blotting**

Protein was isolated by using radioimmune precipitation buffer (containing 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS) with protease inhibitor cocktail (Sigma) on ice for 1 hour and then centrifuged for 20 min at 13,000 g. Supernatant was collected and protein concentrations were measured by the Bradford method (Bio-Rad). Fifty micrograms of protein were dissolved in sample buffer and boiled for 5 min
prior to loading onto an 8% acrylamide gel. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween (TBST), and incubated with rabbit anti-polyclonal antibody to ER (0.4 mg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 hours at 1:500. After washing with TBST, blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence ECL kits (Amersham Bioscience, Little Chalfont, UK).

**MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromide) Assay**

MCF-7 cells were seeded in 96-well plates at a density of $5 \times 10^3$ cells/well in phenol red-free RPMI-1640 containing 5% CD-FBS. Next day, cells were treated with E2 (10 nM) or NP (200 µg/ml) as indicated after media change. Stock MTT solution (50 µl, 2 mg/ml in PBS) was added to each well. After incubation of 3–4 hours at 37°C in humidified CO2 incubator, aspirate the medium and MTT, and then 200 µl of DMSO was added to dissolve MTT-formazan crystals and measured OD in 540 nm.

**Statistical Analysis**

Values shown represent mean ± SD. Statistical analysis was performed by student’s t-test with a p value of less than 0.05 being considered statistically significant.

**Results**

**NP Induces Expression of PR and pS2 in Human Breast Cancer MCF-7 Cells**

The effects of NP on endogenous estrogen-responsive PR and pS2 genes were examined. After treatment of MCF-7 cells with the compounds for 24 hours, steady-state mRNA levels were measured by RT-PCR of total RNA, as indicated in Figs. 1a and 1b. Constitutively expressed human β-actin mRNA was used as an internal control. NP increased steady-state mRNA levels of the PR and pS2 genes after 24 hours of treatment, as did E2 (Figs. 1a and 1b). Co-incubation with 1 µM ICI efficiently blocked the expression of PR and pS2 gene, indicating activation through ER. These data indicate that NP is capable of activating an estrogen-receptor-mediated genomic pathway.

**NP Down-Regulates ERα Protein and mRNA Expression in MCF-7 Cells**

E2 is known to down-regulate the levels of ERα in the breast cancer cell line. To evaluate the effect of NP on the expression of ERα, we have examined ERα protein and mRNA levels in MCF-7 cells by Western analysis and RT-PCR assays. ERα protein levels were down-regulated 60% after 24 hours of E2 and down regulated 80% after NP (200 µg/ml) treatment, as compared to the control (Fig. 2a).

We also have examined the ERα mRNA levels in MCF-7 cells. The ERα mRNA levels were down-regulated after 24 hours of either E2 or NP treatment compared to the
Figure 1. Effects of NP on endogenous estrogen-responsive PR and pS2 mRNA levels. The semi-quantitative RT-PCR for PR and pS2 in the MCF-7 cells shown here is a representative of the 3 independent experiments. (a) The pS2 amplification product was detected at 365 bp. (b) The PR amplification product was detected at 271 bp. Cells were exposed to E2 (10 nM), NP (200 µg/ml), or were in combination with ICI (1 µM) for 24 hours.

Figure 2. Effects of NP on endogenous estrogen-responsive ER mRNA and protein levels. (a) The Western blot analysis shown here is a representative of two independent experiments. Cells were treated with E2 (10 nM) or NP (200 µg/ml) for 24 hours. ER protein was detected at 62 kDa (left). Equal loading of protein in each lane was confirmed by β-actin protein (43 kDa). ER densitometry values are expressed as a percentage of the control (right). (b) The semi-quantitative RT-PCR for PR, pS2, and ER in the MCF-7 cells shown here is representative of the 3 independent experiments. The ERα amplification product was detected at 620 bp.

Co-incubation with 1 µM ICI efficiently blocked the down-regulation of ERα mRNA expression, indicating ERα mRNA down-regulation through ER (Fig. 2b). These results indicate that NP contains an estrogenic effect.
NP Protects against Proliferation of MCF-7 Cell

E₂ is known to stimulate cell growth in breast cancer cells as well. To examine the biological activity of NP, its effects on MCF-7 cell proliferation were determined by using a MTT assay. Treatment of E₂ increased cell proliferation. However, treatment with 200 µg/ml NP inhibited cell proliferation. NP had no toxic effect on the cells (Fig. 3a). NP in the range between 20 and 200 µg/ml significantly inhibited cell growth in a dose dependent manner (Fig. 3b). In addition, the incubation of cell with NP (200 µg/ml) for various periods of time revealed that cell growth was initially detected 48 hours after NP treatment, more evident at 72 hours (Fig. 3c). These results indicate that NP protects against breast cancer cell proliferation.

Discussion

Until recently, hormone replacement therapy was used for its effects against heart disease, osteoporosis, Alzheimer’s disease, and hot flashes, although studies supporting its efficacy are conflicting (Woo et al., 2003). However, such uses of estrogens are associated with side-effects, such as increased risks of breast and endometrial cancers. Alternative medical therapy using plant-derived estrogens known as phytoestrogens has attracted a great deal of attention from the media, medical community, government agencies, and the public (Glazier and Bowman, 2001). Phytoestrogens are a diverse group of nonsteroidal plant compounds that can behave as estrogens by binding to both types of estrogen receptor, ERα receptors and the more recently discovered ERβ receptors (Thompson et al., 1991; Setchell, 1998). However, despite their ability to bind to the estrogen receptors, their effects are much weaker than those of 17β-estradiol, with 10² to10⁵ times less activity (Price and Fenwick, 1985). Even though they have low ER affinity, animal studies have demonstrated in vivo effects probably because they are frequently present in the body in much higher quantities than endogenously produced estrogens (Adlercreutz et al., 1993). Some researchers have also demonstrated progesterone receptor activity of phytoestrogens (Zava et al., 1998). However, it is somewhat disappointing that several previously reported clinical data do not fully support in vitro and animal based in vivo experiments showing effects of herbal agents on menopausal symptoms (Carroll, 2006; Haimov-Kochman and Hochner-Celnikier, 2005). This may be due to many reasons such as insufficient trial size or inadequate design of the trial. A recent review raised a good point that complementary agents should not be limited to a single herb or plant product. Use in combination may be needed for optimal efficacy considering that traditional Chinese medicine has been used as a mixture (Chen et al., 2008).

Here in this study, we evaluated the estrogenic activity of wild yam extract, which contains steroidal saponins, including diosgenin, and has been claimed to influence endogenous steroidogenesis (Komesaroff et al., 2001). The estrogenic effect of wild yam has been hypothesized but no clear in vitro data has been shown. We have found that wild yam activates ER-mediated genomic pathway and down-regulates ERα protein and mRNA through the ER. Our in vitro data support the in vivo results of others (Wu et al., 2005; Aradhana et al., 1992; Higdon et al., 2001) which implicated estrogenic activity of wild yam.
Figure 3. Effects of NP on the MCF-7 cell growth. MCF-7 cells were plated and grown in RPMI-1640 supplemented with 5% CD-FBS. (a) Cells were exposed to E2 (10 nM) or NP (200 µg/ml) for 72 hours. (b) Cells were treated with various concentrations of NP as indicated for 72 hours. (c) MCF-7 cells were treated with NP (200 µg/ml) for the indicated time points and analyzed. The data are representative of at least 3 independent experiments performed 4 times with similar results. All data are expressed as mean ± SEM. Asterisks denote a statistically significant differences compared to the corresponding control value (***p < 0.005, *p < 0.05).
Phytoestrogens have potential anticarcinogenic activities. Early studies focused particularly on their potential ability to reduce the risk of breast cancer, but studies have found that their actions are not purely estrogenic, and their nonhormonal activities could be more important in cancer prevention (Barnes et al., 1995). Our study shows that wild yam extracts also have potential anticarcinogenic activity through inhibition of proliferation of breast cancer cells. Our further efforts would concentrate on in vivo estrogenic activity of the extracts.

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References


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