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Stimulation of Endometrial Glandular Cells with Genistein and Daidzein and their Effects on ERα- and ERβ-mRNA and Protein Expresion

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Abstract. *Phytoestrogens seem to have estrogen-like effects in the human body as their structure is very similar to those estrogens produced in human glands. The aim of the present study was to analyse the effects of genistein and daidzein on estrogen receptor (ER)α- and ERβ-mRNA and protein expression in the endometrium of premenopausal women. Materials and Methods: Glandular endometrial cells were isolated from endometrial biopsies obtained from regularly menstruating women undergoing gynaecological abrasio or hysterectomy. Cells were stimulated with single doses of genistein or daidzein. ERα- and ERβ-protein expression were determined by immunocytochemical analysis. In addition ERα- and ERβ-mRNA expression were determined by quantitative real-time RT-PCR. Quantification was carried out by the $\Delta\Delta C_T$-method using glyceraldehyde phosphate dehydrogenase (GAPDH) as housekeeping gene. Results: Endometrial glandular cells responded to stimulation with genistein and daidzein by alteration of both ERα- and ERβ-mRNA expression. The effects were time- and dose-dependent. Detection of ERα- and ERβ-protein expression by immunocytochemistry showed a dose-dependent regulation after stimulation of isolated endometrial cells with phytoestrogens. Discussion: According to our results, we suggest that ER expression in endometrial glandular cells is regulated by genistein and daidzein on the mRNA and protein levels. We could detect a decrease in ERα- and an increase in ERβ-mRNA expression after stimulation with tested phytoestrogens. Our results are in line with findings that phytoestrogens act as anti-estrogens in organs expressing more ERα and as estrogens in ERβ-presenting organs.*

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Phytoestrogens can be isolated from plants, *e.g.* soy bean, flax seed or elder flower, and are divided into lignans and isoflavones. Both of them seem to have estrogen-like effects in the human body as their structure is very similar to those estrogens produced in human glands. Phytoestrogens bind to both types of estrogen receptors (ER) (α and β) but with a higher affinity to ERβ than steroidal estrogens (1). The most commonly investigated isoflavones, genistein and daidzein, are found in legumes such as soy, chickpeas, clover, lentils, and beans (2). The lignans are found in flax seed, lentils, whole grains, beans, fruits and vegetables. In *in vitro* tests, phytoestrogens showed anticarcinogenic activity. The proposed mechanisms by which they may inhibit cancer cells include the following: inhibition of DNA topoisomerase, suppression of angiogenesis, induction of differentiation in cancer cell lines and induction of apoptosis (3). *In vivo* animal experiments have demonstrated that phytoestrogens can inhibit tumours (4). In a comprehensive review on the potential of phytoestrogens to reduce tumour growth, Fournier *et al.* (5) noted that the addition of soy products reduced tumour incidence or multiplicity in different tumour models. Many studies focused on the isoflavone genistein, which seems to be the primary anticancer soy constituent. The endometrium is the innermost tissue layer of the uterus and mainly consists of two cell types: glandular cells and stromal cells situated between the glandular cells. It is influenced by different hormones which are cycle-dependent. The incidence of endometrial cancer is increased after therapy of breast cancer by tamoxifen, which is a non-steroidal substance with structural similarities to phytoestrogens like genistein, and daidzein. Tamoxifen has an anti-estrogen-like effect in breast, but an estrogen one in endometrial cells (6). Phytoestrogens influence the development of gynaecological cancers (7) and, therefore, the aim of our present study was to analyse the effects of genistein and daidzein on ERα- and ERβ-mRNA and protein expression after stimulation of

Table I. *Monoclonal antibodies against ERα/β.*

Antibody	Clone	Type	Dilution	Source of supply
ERα	1D5[1]	IgG_1, Kappa	non-diluted	Dako
ERβ	PPG5/10	IgG 2a	1/10 with PBS	Serotec

Table II. *Primer and probe sequences and length of PCR fragments.*

Primer/probe	Sequence 5'-3'	Fragment
ERα 5'	TGATTGGTCTCGTCTGGCG	128bp
ERα 3'	CATGCCCTCTACACATTTTCCC	
ERα-probe	TGCTCCTAACTTGCTCTTGGA CAGGAACC	
ERβ 5'	GGTCCATCGCCAGTTATCACAT	227bp
ERβ 3'	GATGCGTAATCGCTGCAGACAG	
ERβ-probe	TGTGAAGCAAGATCGCTAGA ACACACCT	

human endometrial glandular cells of premenopausal women with these substances.

Materials and Methods

Isolation of endometrial glandular cells. Samples of human endometrium in proliferative phase were obtained from 5 premenopausal, non-pregnant women undergoing gynaecological abrasio or hysterectomy for benign diseases. The patients were between 40 and 46 years old, menstruated regularly and normally, and had not undergone hormonal treatment or used an intrauterine device over the previous 3 months. None of the patients had endocrinological problems. Endometrial glandular cells were separated by collagenase digestion, filtration, sedimentation and Ficoll-gradient centrifugation, as previously described (8).

Cell culture. Isolated endometrial glandular cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL Life Technologies, Paisley, Scotland) with 10% inactivated foetal calf serum (FCS) (Sigma, Taufkirchen, Germany), 1% penicillin/streptomycin (Sigma) and 0.5% Amphotericin B (Sigma), in a humidified atmosphere at 37˚C with 5% CO_2. For stimulation, the cells were incubated with genistein and daidzein for different times (0.5, 1, 2, 4, 8 and 48 hours) with various substance concentrations (genistein: 0.115 μmol/ml, 1.15 μmol/ml, 2.3 μmol/ml and daidzein: 0.123 μmol/ml, 1.23 μmol/ml, 2.4 μmol/ml).

Immunocytochemistry. In order to determine ERα- and ERβ-protein expression by immunocytochemical analysis, cells were grown on 3-well multitest-slides to subconfluency. The cells were incubated with single doses of genistein and daidzein for 48 hours. Untreated cells were used as controls. Immunocytochemical reactions were accomplished using the Vectastainβ Elite ABC-Kit (Vector Laboratories, Burlingame, USA). After incubation with monoclonal antibody against ERα/β (Table I), the cells were incubated with biotinylated anti-mouse antibody, followed by visualisation with avidin/biotin-POD and diaminobenzidine. Counter-staining was done with Meyer haemalaun.

RNA extraction. Isolated endometrial glandular cells were adjusted to a cell density of 3 x 10^5cells/ml. The cell suspension (0.8 ml/well) was incubated in a 24-well plate in humidified 5% CO_2 at 37˚C in the presence of the above-stated concentrations of genistein and daidzein. Untreated cells were used as controls. Total RNA was extracted by RNeasy® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol, after the designated times. Purified RNA was quantified and evaluated for purity by UV spectrophotometry.

RT-PCR procedure. TaqMan® EZ RT-PCR Kit (PE Applied Biosystems, Weiterstadt, Germany) was used for reverse transcription and amplification of isolated RNA. Production of cDNA and PCR-amplification was carried out in a single-tube, single-enzyme system without the addition of subsequent enzymes or buffers. All RT-PCR reactions were performed in quadruplicate in optical 96-well reaction microtiter plates covered with optical caps, in a volume of 25 μl containing 1xTaqMan EZ-buffer, 3 mM of $Mn(Oac)_2$, 300 μM of dATP, dCTP, dGTP, 600 μM of dUTP, 2.5 U of rTth DNA-polymerase, 200 nM of each primer, 100 nM of probe, 0.25 U of UNG and 20 ng of RNA/tube. Table II lists the primer and probe sequences and the length of PCR fragments (9). Thermal cycling conditions were: 2 minutes at 50˚C, 45 minutes at 60˚C, 10 minutes at 95˚C, followed by 45 cycles of amplification with 20 seconds at 94˚C and 1 minute at 60˚C and final extension for 10 minutes at 60˚C. The ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems) was used for performing the PCR assays. Quantification was carried out by the $\Delta\Delta C_T$-method using glyceraldehyde phosphate dehydrogenase (GAPDH) as housekeeping gene (GAPDH-control-reagent) (PE Applied Biosystems).

Results

Glandular cells were isolated from endometrium in the proliferative phase and sensitivity to phytoestrogens was tested. By means of immunocytochemistry, ERα- and ERβ-proteins in the endometrial glandular cells were detected. The cells showed higher protein expression of ERα than of ERβ and the expression of the receptor proteins could be stimulated with the tested phytoestrogens. In comparison to untreated controls, a stronger expression of ERα-proteins was found after stimulation with genistein at a concentration of 1.15 μmol/ml and daidzein at a concentration of 1.23 μmol/ml (Figures 1, 2, 3). Also ERβ-proteins showed a higher level after stimulation with daidzein at a concentration of 0.123 μmol/ml (Figures 4, 5).

ERα- and ERβ-mRNA expression in isolated endometrial cells (proliferative phase) was determined by quantitative RT-PCR procedure. Depending on the target amount, positive fluorescence signals were detected after certain PCR cycles (threshold cycle, C_T). ERα-mRNA

Figure 1.

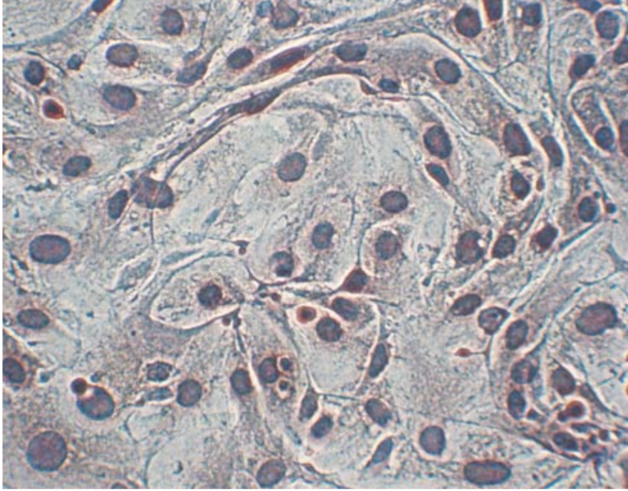


Figure 2.

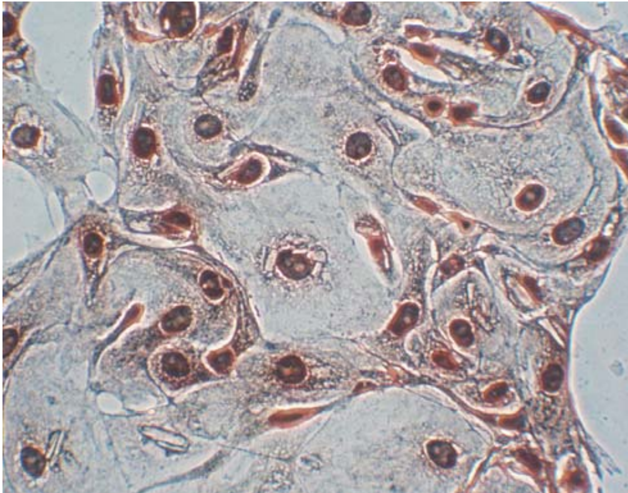
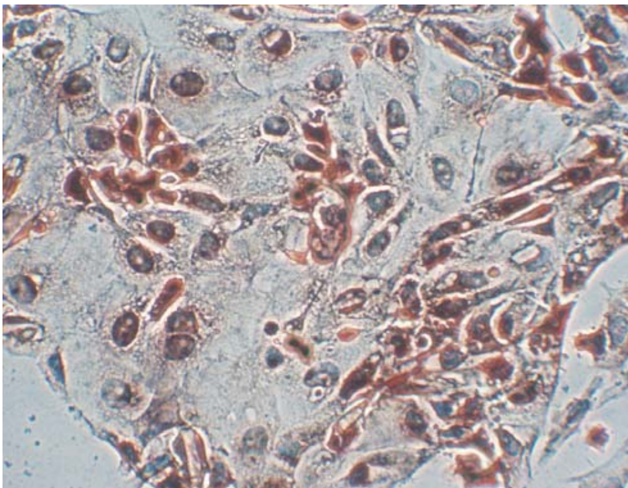


Figure 3.



Figures 1-3. *ERα-protein expression in endometrial glandular cells after cultivation for 48 hours, magnification 400x, 1) control, 2) with genistein (1.15 μmol/ml), 3) with daidzein (1.23 mmol/ml).*

Figure 4.

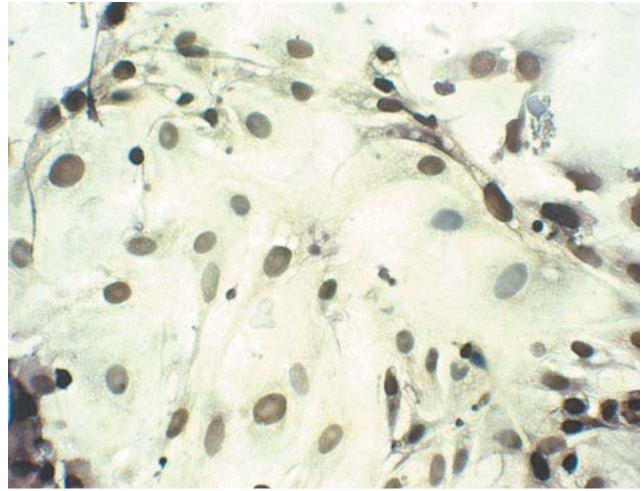
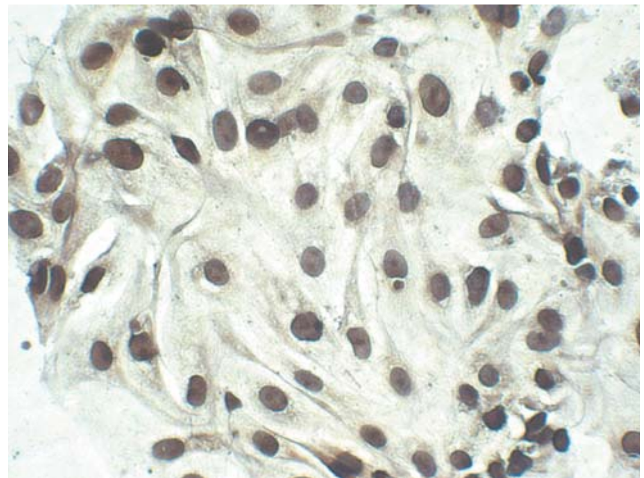


Figure 5.



Figures 4 and 5. *ERβ-protein expression in endometrial glandular cells after cultivation for 48 hours, magnification 400x, 4) control, 5) with daidzein (0.123 μmol/ml).*

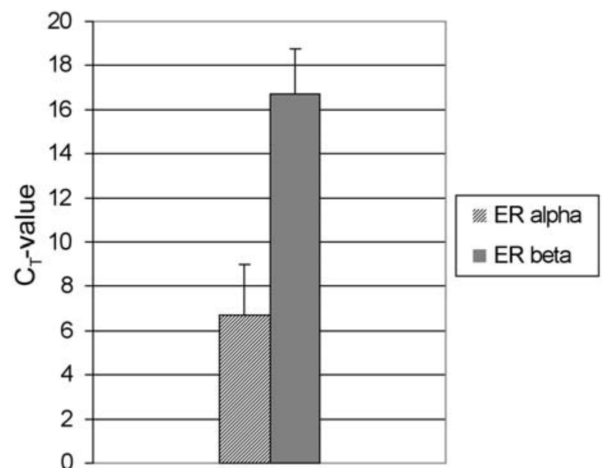


Figure 6. *Expression of ERα- and ERβ-mRNA in endometrial glandular cells after cultivation for one hour.*

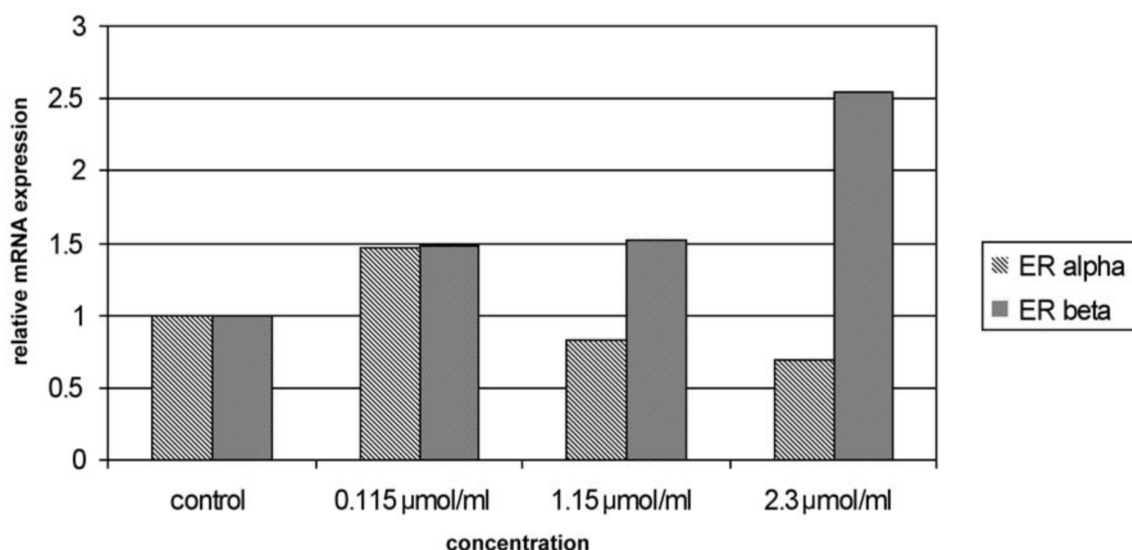


Figure 7. *Expression of ERα- and ERβ-mRNA in endometrial glandular cells after incubation with genistein at different concentrations for one hour.*

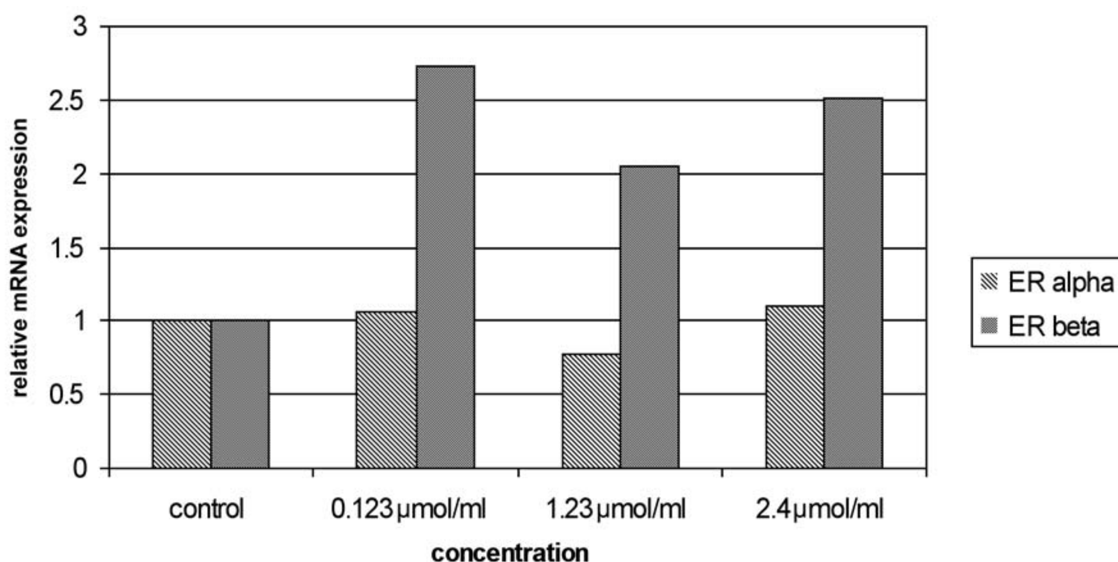


Figure 8: *Expression of ERα- and ERβ-mRNA in endometrial glandular cells after incubation with daidzein at different concentrations for one hour.*

expression was higher than that of ERβ in these cells according to lower C_T values for ERα than ERβ after standardisation to GAPDH (Figure 6).

The optimal incubation time for detection of ER-mRNA copy number alterations by phytoestrogens was evaluated by stimulation of cells with genistein (1.15 µmol/ml) and daidzein (1.23 µmol/ml) for 0.5, 1, 2, 4 and 8 hours. Following RNA extraction, RT-PCR amplification of the ER gene fragments and GAPDH control relative ER-mRNA copy numbers were determined by standardisation to GAPDH for each sample using the $\Delta\Delta C_T$-method. The greatest alterations resulted after one hour with a decrease of both ER-mRNA levels. Genistein treatment down-regulated the expression of ERα- and ERβ- mRNAs after one hour to 75.8% and 32.9% of control values, and with daidzein-incubation to 56.3% and 50.5% of control values (data not shown).

One-hour-stimulation of endometrial glandular cells with different concentrations of phytoestrogens resulted in the following expression patterns of ERα- and ERβ-mRNA:

stimulation with genistein at low concentration first increased the ERα/β-mRNA expression to 146% and 148% of control values. Higher concentrations showed a decrease of ERα-mRNA expression and a rising trend of ERβ-mRNA expression. The highest concentration led to an increase to 255% of the ERβ-mRNA expression of control values (Figure 7). The effect of daidzein led to a relatively non-influenced ERα-mRNA expression and a dose-dependent increase of the ERβ-mRNA expression (Figure 8).

Discussion

Phytoestrogens are known to have a chemical structure similar to those of human estrogens and have been found to bind to estrogen receptors (10). Phytoestrogens bind to both types of estrogen receptors (ERα and ERβ), but with an even higher affinity to the ERβ than steroidal estrogens (1). In epidemiological studies, a lower risk of developing breast or endometrial cancer was demonstrated among Asian women, known to have higher intake of phytoestrogens through soy-rich food (11-13). Addition of soy protein, the main source of isoflavones, to a special diet significantly decreased chemically- induced mammary cancer in rats (14). Furthermore, isoflavones have been shown to down-regulate ERα expression in breast cancer cell lines (15). Isoflavones inhibit the activity of enzymes involved in estrogen metabolism, like aromatase and enzyme for 17β-oxidoreduction of estrogens *in vitro*. Several studies directly examined the effects of isolated isoflavones on mammary tumorigenesis (16). Plant-derived estrogens may cause estrogenic as well as anti-estrogenic effects on metabolism. An important anti-estrogenic substance used in therapy and prevention of ER-positive breast cancer is tamoxifen. Tamoxifen is a non-steroidal triphenylethylene-derived anti-estrogen and acts through the classic ER, ERα. On the other hand, most benign endometrial pathologies obtained from postmenopausal tamoxifen-treated women support an estrogen-like effect on human endometrium (17), leading to a higher incidence of endometrial cancer within/after tamoxifen therapy (18). Endometrial cells express a higher level of ERα than ERβ, the latter being down-regulated in endometriotic cysts (9). With this background, the analysis of the influence of phytoestrogens on hormone receptor expression in human endometrium is interesting for the development and safety of therapy with such plant-derived estrogens.

Our investigations on endometrial glandular cells showed that genistein and daidzein caused changes to the expression of ERα- and ERβ-mRNA and the protein level. During the proliferative phase of the menstrual cycle, these receptors reach a maximum (19). The protein expression of ERα after stimulation of cells with phytoestrogens shows similar results, as already described by Mylonas *et al.* (8). The ERβ-protein expression was increased as well. The effects of genistein and daidzein on ERα/β-mRNA expression were time- and dose-dependent. Stimulated cells adapted to a changed concentration of phytoestrogens within one hour. Binding of genistein and daidzein to present receptors caused signals inside cells that changed the ERα/β-mRNA expression in favour of ERβ. The higher response of ERβ with regard to stimulation of mRNA expression can be explained by the stronger affinity of phytoestrogens for ERβ in comparison to ERα (10). According to our results, we suggest that ERα/β expression in endometrial glandular cells may be regulated by phytoestrogens at the mRNA and protein levels. Interestingly, ERβ-mRNA expression was stimulated by genistein and daidzein, suggesting protective properties against endometrial cancer. Our findings are in line with studies underlying the osteoprotective effect of phytoestrogens on bone (20-22), which is known to express high levels of ERβ. On the other hand, carcinoma prevention effects could also be mediated through ER-independent pathways (23). Furthermore, it should be taken into account that the benefits of phytoestrogens in cancer therapy and prevention depend on their bioavailability through colonic microflora (24).

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