INTRODUCTION

Soy isoflavones such as genistein, daidzein and biochanin A are potential endocrine disruptors in domesticated herbivores e.g. pigs (1, 2) and are used as natural estrogen replacement therapy for postmenopausal women (3, 4). In comparison to genistein, daidzein is a poorly characterized phytoestrogen (4, 5). Preliminary reports revealed that daidzein altered steroid hormone concentrations in the plasma of premenopausal women (6, 7) and pregnant rats (8). A possible indirect (i.e. involving gonadotropins) effect of daidzein on steroidogenesis was suggested in miniature pigs (9). Recently, an inhibitory action of daidzein on progesterone (P4) secretion and possible involvement of 3β-hydroxysteroid dehydrogenase (3β-HSD) was demonstrated in porcine granulosa cells (10).

The structure of isoflavones enables them to bind to the estrogen receptor (ER) and recent studies revealed that their binding affinity for ERβ is significantly higher than for ERα (11-13). However, individual isoflavones exhibit different affinity for each of the two ER isoforms (14). Moreover, chronic exposure to dietary isoflavones may change ER expression in reproductive tissues including ovaries (15, 16).

Like other phytoestrogens, daidzein binds to ER, especially to ERβ (17). Treatment with dietary daidzein decreases expression of both ERα and ERβ mRNA in the mouse ovary (16) as well as ERβ mRNA in porcine hypothalamus (13). The objectives of the study were to compare the in vitro effects of daidzein or 17β-estradiol (E2) on: 1) progesterone (P4) secretion by luteinized granulosa cells harvested from large porcine follicles, as well as 2) estrogen receptor α and β (ERα and ERβ) mRNA and protein expression in the cells. In addition, the effect of daidzein on E2 secretion and viability of the granulosa cells was examined. We found that basal and gonadotropin-stimulated P4 secretion were inhibited in granulosa cells cultured in the presence of daidzein either for 24 or 48 hours. In contrast to daidzein, E2 reduced P4 secretion only during 24-hour cell cultures. Daidzein did not affect E2 secretion by granulosa cells. The expression of ERα and ERβ mRNA, as well as ERβ protein, was up-regulated by daidzein but unaffected by E2. To conclude, the soy estrogen daidzein acts directly on the porcine ovary to decrease progesterone production and to increase expression of ERβ mRNA and protein. Daidzein actions in porcine luteinized granulosa cells differ from those of estradiol and it may suggest disadvantageous effects of the phytoestrogen on reproductive processes in females.

KEYWORDS: daidzein, estradiol, progesterone, gonadotropins, phytoestrogens, granulosa cells, steroidogenesis, ERα expression, ERβ expression

MATERIAL AND METHODS

CHEMICALS

Daidzein, cell culture supplies, medium M199, nystatin, red blood cell lysis buffer, trypan blue solution, E2, P4, and luteinizing hormone (LH) were obtained from Sigma (USA). Labeled [2,4,6,7-3H] 17β-estradiol and [1,2,6,7-3H] progesterone were purchased from Amersham Pharmacia Biotech (Great Britain). Eagle’s medium and calf serum (CS) were used from Biomed (Poland), bovine serum albumin (BSA) from ICN Biomedicals (USA), gentamycin from KRKA (Slovenia) and culture plates from Becton Dickinson Labware Europe (France). The cell viability test was performed with alamarBlue dye (BioSource International, Belgium). For total JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2009, 60, 2, 95-105 www.jpp.krakow.pl

A. NYNCA1, O. JABLONSKA1, M. SLOMCZYNSKA2, B.K PETROFF3, R.E. CIERESZKO1

EFFECTS OF PHYTOESTROGEN DAIDZEIN AND ESTRADIOL ON STEROIDOGENESIS AND EXPRESSION OF ESTROGEN RECEPTORS IN PORCINE LUTEINIZED GRANULOSA CELLS FROM LARGE FOLLICLES

1Department of Animal Physiology, University of Warmia and Mazury, Olsztyn, Poland; 2Department of Endocrinology and Tissue Culture, Institute of Zoology, Jagiellonian University, Krakow, Poland; 3Center for Reproductive Sciences, University of Kansas Medical Center, Kansas City, USA
RNA isolation from cells TRIzol Reagent (Invitrogen, USA) was used and for RT-PCR Omniscript Reverse Transcription Kit and HotStarTaq Master Mix Kit (Qiagen, USA). The VECTASTAIN ABC System were obtained from Vector Laboratories (USA) and 3,3′-diaminobenzidine from Dako (Denmark).

Cell cultures and experimental design

Porcine ovaries with large, preovulatory (≥ 8 mm in diameter) follicles were collected in a local slaughterhouse (Krokowo/Jeziorany, Poland) and transported promptly in cold buffered physiological saline (PBS) supplemented with gentamycin and nystatin. Granulosa cells were isolated from the ovaries using a modification (19) of the method described by Stoklosowa et al. (20). All stages of experiments were performed in sterile conditions. Ovaries with follicles were placed in beaker containing M199 medium supplemented with 5% bovine serum albumin (BSA), gentamycin and nystatin. Follicles were dissected from ovaries in Petri dishes containing small amount of M199 medium. Using pair of fine forceps, theca interna/granulosa layers were separated from external layers of follicular wall. Granulosa cells attached to the theca interna layer were rinsed off by intensive pipetting (10 s) and supernatant containing granulosa cells was decanted. The cells were washed (M199 medium) and decanted several times until supernatant became transparent. Then, granulosa cells were rinsed in M199 medium with 5% BSA and centrifuged (180×g, 10 min, 18°C). The cell pellet was treated with red blood cells lysing buffer and washed another three times in M199 medium. After the last centrifugation, the granulosa cells were resuspended in fresh M199 medium and counted in Burk’s chamber. Cells viability was determined by 0.4% trypan blue dye exclusion and it was ≥ 97%. Incubation medium was Eagle’s medium containing gentamycin (0.05 mg/ml) and nystatin (120 µ/ml). Aliquots of granulosa cells were cultured in: 1/ 96-well plates, 0.2×10⁵ cells per well (to measure cell viability), 2/ 24-well plates, 1.5×10⁵ cells per well (to measure steroid secretion and to amplify PCR products), and 3/ 6-well plates, 2×10⁶ cells per well (to analyze the level of ER mRNAs). Following 48-72 hours of attachment (37°C, 10% CS, 95% air/5% CO2), cells were preincubated for 72 h and then cultured for 24 hours. The experiments were terminated the media (-20°C) and/or and/or LH (100 ng/ml) and/or LH (100 ng/ml). In all experiments medium without treatments served as control. Concentration of steroid hormones in medium was measured by a previously validated H-RIA (22, 23, 24). Intra- and inter-assay coefficients of variation for Pr were 3.75 and 2.45%, respectively. Intra- and inter-assay coefficients of variation for E2 were 3.1 and 2.25%, respectively. Sensitivities of the Pr and E2 assays were 6 and 1 pg/tube, respectively, and not altered by treatment. Serial dilutions of medium samples showed parallelism with the standard curves of examined steroids. All analyses were performed in triplicates.

The effect of daidzein and E2 on ERα and ERβ mRNA expression

ERα and ERβ mRNA expression levels were measured in luteinized granulosa cells preincubated for 48 hours and then cultured for subsequent 48 hours with or without daidzein (0.5 and 5 µM) or E2 (37 and 370 nM). Total RNA was extracted from the granulosa cells after the culture using TRIzol Reagent. For cDNA synthesis, 1 µg of RNA was reverse transcribed in a 20 µl reaction volume with 0.5 µg oligo(T)18 primer (Roche, Germany) using the Omniscript RT Kit (Qiagen, USA). Complementary DNA was amplified by polymerase chain reaction (GeneAmp PCR System 2400, Perkin Elmer, USA) in a total volume of 50 µl using 40 pmol of porcine ERα or ERβ sense and antisense primer pairs (Table 1). To provide an appropriate internal control, coamplification of GAPDH was carried out in each sample using the GAPDH-sense and antisense primer pair (Table 1). Amplification tubes also contained 5 µl of the first strand cDNA, 25 µl of HotStarTaq Master Mix (2.5 U HotStarTaq DNA Polymerase, 1×PCR buffer containing 1.5 mM MgCl₂, 200 µM of each dNTP). The optimal number of cycles, ensuring the termination of amplification for the genes in the log phase was established by primer dropping method (25): 1) 40 and 30 cycles were employed for ERα and GAPDH, respectively, and 2) 38 and 26 cycles were employed for ERβ and GAPDH, respectively. PCR reactions were performed under the following cycling conditions: initial denaturation at 95°C for 15 min, an appropriate number of cycles including denaturation at 95°C for 20 s, annealing at 59°C (for ERα gene) or 58°C (for ERβ gene) for 30 s and elongation at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Negative controls were performed without reverse transcriptase and for each pair of primers the non template controls were carried out.

Aliquots of PCR reaction products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination. Gel images were saved by FOTO/Analyst Achiever software (Fotodyne, USA) and product yield was determined using GelScan for Windows ver.1.45 software (Kucharczyk, Poland). Data were expressed as a ratio of ERα or ERβ mRNA relative to GAPDH mRNA in arbitrary optical density units (OD).

In addition, the PCR-amplified DNA was sequenced (by DNA Sequencing and Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland) in both directions to confirm the accuracy of amplification. Comparison of the PCR-amplified DNA sequence to that in the database indicated 100 and 99% homology for ERα and ERβ, respectively, at the nucleotide level.

The effect of daidzein and E2 on steroid hormone secretion

For estimation of Pr and E2 secretion, granulosa cell monolayers were preincubated for 72 h and then cultured for 24 or 48 h with or without daidzein (0.05, 0.5, 5, 50 µM) in the absence or presence of LH (100 ng/ml). Separate cell cultures were used to examine the two incubation times. To compare the effects of daidzein and endogenous estrogen (E2) on Pr production, cells were also incubated with E2 [1 (3.7), 10 (37), or 100 (370) ng/ml (nM)] and/or LH (100 ng/ml). In all experiments medium without treatments served as control. Concentration of steroid hormones in medium was measured by a previously validated H-RIA (22, 23, 24). Intra- and inter-assay coefficients of variation for Pr were 3.75 and 2.45%, respectively. Intra- and inter-assay coefficients of variation for E2 were 3.1 and 2.25%, respectively. Sensitivities of the Pr and E2 assays were 6 and 1 pg/tube, respectively, and not altered by treatment. Serial dilutions of medium samples showed parallelism with the standard curves of examined steroids. All analyses were performed in triplicates.
Table 1. Sequences of primers used for PCR, the length of PCR products and the Genbank access numbers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Size of PCR product</th>
<th>Access number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>5' AGGGAGAGCTTTGTTGAGG 3'</td>
<td>305 bp</td>
<td>AF0357757</td>
</tr>
<tr>
<td>ERβ</td>
<td>5' GCTTCGTTGAGCAGCTGTTG 3'</td>
<td>262 bp</td>
<td>AF164957</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5' ATGGTGAAAGTCGGAGTGA 3'</td>
<td>681 bp</td>
<td>AF017079</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of daidzein on granulosa cell viability (mean±SEM) assessed by alamarBlue™ test (n=4). Following preincubation (48 h), cells were cultured for 48 h (0.2×10^5 cells/100 µl) with daidzein (0.05-50 µM) and staurosporin (STS; 5 µM; a positive control). Bars without common superscripts designate significant differences (p<0.05).

Statistical analysis

Analyses were performed using Statistica program (StatSoft Inc., USA). The raw hormone concentrations data were log transformed and then statistically analyzed. Data expressed as a percentage of the number of stained cells were arcsin transformed and then statistically analyzed. Data expressed as a percentage of the number of stained cells were arcsin transformed before the statistical analyses. Amounts of P4 secreted in the absence (control) and presence of LH were compared by Student’s t-test. All other data were analyzed by one-way ANOVA for repeated measurements followed by least significant difference (LSD) post hoc test. The level of significance was set at p<0.05 for all analyses. RIA data are expressed as a percentage of control culture (100%); all other data are presented as mean±SEM.

RESULTS

The effect of daidzein on the viability of granulosa cells

Daidzein at concentrations ranging from 0.05 to 50 µM did not affect the viability of porcine granulosa cells harvested from large, preovulatory follicles (Fig. 1). A significant decrease (p<0.05) in the cell viability was, however, seen when a positive control (5 µM staurosporin) was used.

The effect of daidzein and E2 on steroid hormone secretion

LH stimulated (p<0.05) P4 secretion by porcine luteinized granulosa cells independently on the culture time (data not shown). Daidzein (0.05-50 µM) inhibited (p<0.05), in a dose dependent manner, basal and LH-stimulated P4 production by luteinized granulosa cells cultured for 24 and 48 hours (Fig. 2). The inhibition of basal and LH-stimulated P4 production was, however, more pronounced after 48 than after 24 hours of culture with daidzein (Fig. 2). A decrease (p<0.05) in P4 secretion was also found in granulosa cells cultured with E2 (3.7-370 nM) for 24 hours (Fig. 3). In contrast, P4 secretion was significantly
Fig. 2. Effect of daidzein on basal and LH-stimulated progesterone secretion by porcine granulosa cells originated from large follicles (n=4). Following preincubation (72 h), the cells were cultured for 24 h (A) or 48 h (B; 1.5×10^5 cells/ml; Eagle’s medium, 5% calf serum) with daidzein (0.5 - 50 µM) or daidzein and LH (100 ng/ml). Data are expressed as a percentage of control culture (100%). One-way Anova for repeated measurements was performed on raw data. Bars without common superscripts designate significant differences (p<0.05).

Fig. 3. Effect of 17β-estradiol (E₂) on progesterone secretion by porcine granulosa cells originated from large follicles (n=4). Following preincubation (72 h), the cells were cultured for 24 h (A) or 48 h (B; 1.5×10^5 cells/ml; Eagle’s medium, 5% calf serum) with E₂ (37-370 nM) or E₂ and LH (100 ng/ml). Data are expressed as a percentage of control culture (100%). One-way Anova for repeated measurements was performed on raw data. Bars without common superscripts designate significant differences (p<0.05).
increased (p<0.05) after 48 hours of culture with E2 (Fig. 3B). Regardless of culture time, no effect of E2 on P4 secretion was observed in the presence of LH (100 ng/ml) (Fig. 3).

Most doses of daidzein did not affect E2 secretion by porcine granulosa cells (Fig. 4). Only the lowest dose of daidzein (0.05 µM) stimulated (in the absence of LH) or inhibited (in the presence of LH) E2 secretion (Fig. 4B).

The effect of daidzein and E2 on ERα and ERβ mRNA expression

Estrogen receptor α mRNA expression was significantly higher (p<0.05) in granulosa cells cultured with 5 µM of daidzein comparing to control. A lower dose of daidzein (0.5 µM) only tended to increase the expression of ERα mRNA (Fig. 5A). In contrast, the mRNA expression of ERα was not affected by E2 (37-370 nM; Fig. 5B).

5 µM daidzein increased (p<0.05) the transcript concentration of ERβ gene in cultured porcine granulosa cells (Fig. 6A). Similarly to ERα, a lower dose of daidzein (0.5 µM) only tended to increase the expression of ERβ mRNA. In contrast to daidzein, no effect of E2 (37-370 nM) on the ERβ mRNA cellular level was found (Fig. 6B).

The effect of daidzein and E2 on ERα and ERβ protein expression

No expression of ERα protein was found in the examined granulosa cells (data not shown) although the protein was clearly detected in porcine uterine slices (data not shown). In contrast, we found that ERβ protein was detectable in nuclei of granulosa cells isolated from large porcine follicles. Figs. 7 and 8 present representative ERβ immunostaining in the porcine granulosa cells cultured in the absence (Figs. 7A and 8A) or in the presence of daidzein (0.05-50 µM; Figs. 7B-E) or E2 (37-370 nM; Figs. 8B-C). Table 2 shows the effect of daidzein on the percentage of the ERβ positively stained granulosa cells and the intensity of cell nuclei immunostaining. The two intermediate concentrations of daidzein (0.5 and 5 µM) caused a significant increase (p<0.05) in the number of positively stained granulosa cells. In addition, there was a tendency to increase the percentage of the stained cells after their treatment with 50 µM (p=0.06) and 0.05 µM daidzein (p=0.08). No significant changes of the intensity of the ERβ immunoreactivity in the granulosa cells were found after daidzein administration to the culture medium (Table 2).

In contrast to daidzein, E2 did not alter the percentage of ERβ positively stained cells or the intensity of the ERβ immunostaining (Table 3).

**DISCUSSION**

Daidzein inhibited basal and LH-stimulated P4 production by porcine luteinized granulosa cells harvested from large (≥ 8 mm) follicles in a dose dependent manner in the current study. This inhibition of P4 was more pronounced with time of exposure of cells to daidzein. Tiemann et al. (10) recently reported that comparable doses of daidzein suppressed basal and forskolin-stimulated P4 secretion by porcine granulosa cells from medium follicles (3-6 mm) cultured for two days in the absence of serum. Thus, the inhibition of porcine granulosal P4 production by daidzein appears to be independent on serum, gonadotropin or forskolin stimulation.

![Fig. 4](image)

**Fig. 4.** Effect of daidzein on 17β-estradiol secretion by porcine granulosa cells originated from large follicles (n=4). Following preincubation (72 h), the cells were cultured for 24 h (A) or 48 h (B; 1.5×10⁵ cells/ml; Eagle’s medium, 5% calf serum) with daidzein (0.05-50 µM) or daidzein and LH (100 ng/ml). Data are expressed as a percentage of control culture (100%). One-way Anova for repeated measurements was performed on raw data. Bars without common superscripts designate significant differences (p<0.05).
Genistein (the most widely studied phytoestrogen) also suppresses basal or gonadotropin-stimulated P₄ production in vitro in several species (1, 10, 19, 22, 26-28). Serum P₄ is decreased in women subjected to genistein- and daidzein-rich diets (6, 7) and in cows fed soy-rich diets (29). Overall, the inhibitory effects of phytoestrogens on ovarian P₄ production are consistent across species and exposure paradigms (1). Such endocrine disruption may be responsible for inadequate

![Fig. 5. Effect of daidzein (A) and 17β-estradiol (E₂; B) on ERα mRNA expression determined by semiquantitative RT-PCR in porcine granulosa cells originated from large follicles (n=4). Upper panels show representative images of agarose gels; MM: molecular marker, D: daidzein, E₂: 17β-estradiol. Lower panels show the results of densitometric analysis of ERα mRNA relative to GAPDH mRNA. Values are expressed as means±SEM of arbitrary optical density units (OD). Bars without common superscripts designate significant differences (p<0.05).](image)

![Fig. 6. Effect of daidzein (A) and 17β-estradiol (E₂; B) on ERβ mRNA expression determined by semiquantitative RT-PCR in porcine granulosa cells originated from large follicles (n=4). Upper panels show representative image of agarose gels; MM: molecular marker, D: daidzein, E₂: 17β-estradiol); lower panels show the results of densitometric analysis of ERβ mRNA relative to GAPDH mRNA. Values are expressed as means±SEM of arbitrary optical density units (OD). Bars without common superscripts designate significant differences (p<0.05).](image)

Table 2. The effect of daidzein on the percentage of the stained granulosa cells and on the intensity of the ERβ immunostaining (mean±SEM) in the granulosa cells harvested from the large porcine ovarian follicles (n=4)

<table>
<thead>
<tr>
<th>DAIDZEIN (µM)</th>
<th>Percentage of the stained cells (%)</th>
<th>Intensity of the immunostaining (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>0</td>
<td>25.7</td>
<td>4.8</td>
</tr>
<tr>
<td>0.05</td>
<td>40.2b</td>
<td>11.6</td>
</tr>
<tr>
<td>0.5</td>
<td>43.1*</td>
<td>11.2</td>
</tr>
<tr>
<td>5</td>
<td>46.5*</td>
<td>6.7</td>
</tr>
<tr>
<td>50</td>
<td>41.7a</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* p<0.05; b p=0.06; a p=0.08
Table 3. The effect of 17β-estradiol on the percentage of the stained granulosa cells and on the intensity of the ERβ immunostaining (mean±SEM) in the granulosa cells harvested from the large porcine ovarian follicles (n=4)

<table>
<thead>
<tr>
<th>17β-ESTRADIOL (nM)</th>
<th>Percentage of the stained cells (%)</th>
<th>Intensity of the immunostaining (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>45.3 ± 7.4</td>
<td>154.4 ± 12.1</td>
</tr>
<tr>
<td>37</td>
<td>48.3 ± 3.7</td>
<td>163.0 ± 7.3</td>
</tr>
<tr>
<td>370</td>
<td>47.8 ± 2.9</td>
<td>159.9 ± 8.3</td>
</tr>
</tbody>
</table>

Fig. 7. Immunoexpression of estrogen receptor β (ERβ) determined by immunocytochemistry in porcine granulosa cells originated from large follicles (n=4). A: cells without treatments, B – E: cells treated with daidzein (0.05-50 µM), F: a negative control; magnification 250×.
follicular growth in animals fed diets enriched with phytoestrogens and may affect subsequent luteal function (1, 10, 15).

Tiemann et al. (10) indicated that inhibition of steroidogenesis by high (> 10 µM) doses of phytoestrogens may be due to cytotoxicity. In the current study, daidzein at concentrations ranging from 0.05 to 50 µM did not affect viability of cultured porcine granulosa cells. These results are in agreement with data reported for daidzein and growing porcine follicles (10). However, genistein at doses of 50 µM and higher decreased viability of granulosa cells obtained from these follicles (10). It appears that cell viability tests should be performed routinely in xenobiotic in vitro studies.

In contrast to the phytoestrogen daidzein, 17β-estradiol suppressed secretion of P4 only at 24 hours of culture. While an increase in P4 secretion was demonstrated after 48 hours. Such duality in E2 action in vitro in porcine granulosa cells was reported previously (30). The effect of daidzein on follicular function in pigs differs from that of endogenous estrogens.

Most doses of daidzein did not affect E2 secretion by porcine granulosa cells in the present study. Similarly, others found that non-toxic concentrations of daidzein did not affect basal E2 secretion by granulosa cells from small (31) and growing porcine follicles (10). Our earlier study showed a lack of genistein effect on secretion of E2 by various granulosa cell populations in pigs (19). A similar lack of effect of phytoestrogens on estrogen synthesis and secretion has been documented for other species (28, 32-35).

In the presence of LH, the lowest concentration of daidzein (0.05 µM) inhibited E2 secretion during 48 h of luteinized granulosa cell culture. Similarly, an inhibitory action of phytoestrogens on E2 production, reported by some researchers, was usually associated with the presence of a stimulatory agent in the medium, a prolonged culture time and/or high doses of phytoestrogens (1, 28, 31, 33-35). In contrast, the basal E2 production was increased by 0.05 µM of daidzein during 48 h of cell culture. Some environmental estrogens were reported to stimulate aromatase activity in human (36; DDE) and porcine (37; DDT and DDE) granulosa cells. It cannot be excluded that the lowest dose of daidzein affected aromatase activity in our experiment. On the other hand, an observed stimulatory action of high and potentially toxic concentrations of phytoestrogens (10, 32, 38) was suggested to result from a leakiness of the granulosa cell membrane (10). With the exception of the one study (10) however, cell viability testing did not accompany the examination of the steroidogenic effect of phytoestrogens.

In view of available data a question why E2 secretion was affected only by 0.05 µM of daidzein and why the cell response was different in the presence or absence of LH cannot be answered easily. Some suggestions may be provided by results of Almstrup
et al. (39) who in their search for aromatase inhibitor found that in MCF-7 cells, a weak phytoestrogen, biochanin A affected expression of estrogen-induced pS2 mRNA in a U-shaped manner. The examined biochanin doses ranged from 1 nM to 10 µM. It would be noteworthy to test how doses of daidzein lesser than 50 nM might affect E2 secretion by porcine granulosa cells.

Daidzein was found to increase the transcript concentrations of both ERα and ERβ genes in granulosa cells from large porcine follicles. There are very limited data concerning intracellular mechanism of phytoestrogen action in ovarian cells. Jefferson et al. (15) demonstrated that expression of the ERα gene was increased and that of ERβ unaltered in ovaries of mice injected with genistein neonatally. This is in contrast to the results of Takashima-Sasaki et al. (16) who found that prenatal and neonatal treatment with dietary daidzein and genistein decreased ovarian expression of ERα and ERβ mRNA in mice. The reported differences in the effects of phytoestrogens on expression of ovarian ER genes, in addition to being species dependent, are probably caused by dissipimilar administration routes and different exposure periods (prenatal, neonatal, adult).

We found that ERβ protein was located predominantly in the nuclei of the granulosa cells of large porcine follicles. In contrast, immunoreactive ERα was not observed in these cells. Similar results were presented earlier by Slomczynska and Wozniak (40) who demonstrated the predominance of ERβ over ERα protein in the porcine ovary. This is also true for other species (41-43). Since ERβ predominates over ERα in the ovary (40, 44-46) and phytoestrogens bind preferably to ERβ (4), the effect of daidzein on ovarian ERβ expression is of special importance. This conclusion is further supported by the upregulation of ERβ by daidzein in this study.

The fact that we did not find ERα protein in porcine granulosa cells but did observe a stimulatory effect of daidzein on ERα mRNA concentration may also have important ramifications for endocrine disruption. Since some actions of estrogens are mediated through ERα exclusively (47, 48), increased expression of ERα mRNA may reflect a blockade of endogenous estrogen signaling by the phytoestrogen daidzein. ERα upregulation by daidzein may also increase the sensitivity of the ovary to endogenous and exogenous estrogens leading to premature luteinization and other disorders. However, in the current study 17β-estradiol did not affect ERα or β mRNA or protein arguing against a competitive mechanism for ER regulation by daidzein here.

In contrast to daidzein and despite the apparent action on P4 secretion, E2 did not affect ER expression in the examined cells. The lack of E2 effect may, however, result from long exposure (48 h) of the cells to estrogen. Sharma et al. (49) demonstrated a biphasic and time-dependent action of E2 on ER immunoeexpression in granulosa cells in rats: nuclear ERα and ERβ staining increased after 1.5 h-24 h but decreased after 48 h of exposure to E2. Hamster follicles exposed to E2 for 24 h exhibited significant increases in protein levels of both ER subtypes (50). The dynamics of changes following the ER activation may vary for different ligands, tissues or species. Moreover, phytoestrogens and E2 differ in their affinity binding to particular ER types (11) as well as their ERE-transactional properties (4). Daidzein and other phytoestrogens may act as natural selective ER modulators (SERM) by recruiting specific co-regulators that trigger ERα and/or ERβ mediated transcription pathways (51) in a different pattern than estradiol.

In summary, the soy estrogen daidzein acts directly on the porcine ovary to decrease progesterone production and to increase expression of ERβ. Daidzein actions in porcine luteinized granulosa cells differ from those of 17β-estradiol and it may suggest disadvantageous effects of the phytoestrogen on reproductive processes in females.

Acknowledgements: This study was supported by the State Committee for Scientific Research as a Solicited Project PBZ-KBN-084/P06/2002 from 2003 to 2005 and UWM No. 0206.805. We thank Dr A.F. Parlow (NIDDK’s National Hormone & Peptide Program) for providing porcine LH.

Conflict of interests: None declared.

REFERENCES


Received: July 31, 2008
Accepted: April 30, 2009

Author’s address: Assoc. Prof. Renata Ciereszko, Department of Animal Physiology, Oczapowskiego 1A, 10-719 Olsztyn, Poland; Phone: 48 89 523 32 01; Fax: 48 89 523 39 37; e-mail: reniac@uwm.edu.pl