Phytoestrogens Modulate Prostaglandin Production in Bovine Endometrium: Cell Type Specificity and Intracellular Mechanisms

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Prostaglandins (PGs) are known to modulate the proper cyclicity of bovine reproductive organs. The main luteolytic agent in ruminants is $PGF_{2\alpha}$, whereas PGE_2 has luteotropic actions. Estradiol 17β (E_2) regulates uterus function by influencing PG synthesis. Phytoestrogens structurally resemble E_2 and possess estrogenic activity; therefore, they may mimic the effects of E_2 on PG synthesis and influence the reproductive system. Using a cell-culture system of bovine epithelial and stromal cells, we determined cell-specific effects of phytoestrogens (i.e., daidzein, genistein), their metabolites (i.e., equol and para-ethyl-phenol, respectively), and E_2 on $PGF_{2\alpha}$ and PGE_2 synthesis and examined the intracellular mechanisms of their actions. Both PGs produced by stromal and epithelial cells were significantly stimulated by phytoestrogens and their metabolites. However, $PGF_{2\alpha}$ synthesis by both kinds of cells was greater stimulated than PGE_2 synthesis. Moreover, epithelial cells treated with phytoestrogens synthesized more $PGF_{2\alpha}$ than stromal cells, increasing the $PGF_{2\alpha}$ to PGE_2 ratio. The epithelial and stromal cells were preincubated with an estrogen-receptor (ER) antagonist (i.e., ICI), a translation inhibitor (i.e., actinomycin D), a protein kinase A inhibitor (i.e., staurosporin), and a phospholipase C inhibitor (i.e., U73122) for 0.5 hrs and then stimulated with equol, para-ethyl-phenol, or E_2. Although the action of E_2 on $PGF_{2\alpha}$ synthesis was blocked by all reagents, the stimulatory effect of phytoestrogens was blocked only by ICI and actinomycin D in both cell types. Moreover, in contrast to E_2 action, phytoestrogens did not cause intracellular calcium mobilization in either epithelial or stromal cells. Phytoestrogens stimulate both $PGF_{2\alpha}$ and PGE_2 in both cell types of bovine endometrium *via* an ER-dependent genomic pathway. However, because phytoestrogens preferentially stimulated $PGF_{2\alpha}$ synthesis in epithelial cells of bovine endometrium, they may disrupt uterus function by altering the $PGF_{2\alpha}$ to PGE_2 ratio. Exp Biol Med 230:326–333, 2005

Key words: cattle; endometrium; phytoestrogens; $PGF_{2\alpha}$; PGE_2

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Introduction

Soy phytoestrogens have been the subject of many reviews that describe their potential health benefits for both humans and animals (1–3). On the other hand, these substances also have some hazardous effects, especially on animals fed pasture that is rich in phytoestrogens (4). Daidzein and genistein are two major phytoestrogens that are present in soy (5, 6). In ruminants, rumen microorganisms convert daidzein into equol and genistein into para-ethyl-phenol (7). There is increasing evidence that phytoestrogens can disrupt the reproductive processes in various species including humans (8, 9), rats (10), and cows (11). Phytoestrogens are also shown to inhibit the secretion of hypophyseal luteinizing hormone in the rat (12). Low levels of luteinizing hormone cause a decrease of progesterone (P4) production which, in turn, leads to high abortion rate (13). The decrease of pregnancy rate can also be attributed to phytoestrogen-dependent inhibition of the production of endogenous estrogens, which leads to disturbances in follicle development (9, 14). Thus, phytoestrogens acting as antagonists or/and agonists of endogenous estrogens may disrupt numerous reproductive processes on several levels of regulation.

In ruminants, endogenous estrogens are known to

control the length of the estrous cycle by influencing prostaglandin (PG) synthesis (see Ref. 15 for a review). For example, the removal of estrogens on Day 8 of the cycle by destroying ovarian follicles with X-irradiation in ewes resulted in prolongation of the estrous cycle and lack of luteolysis (16). On the other hand, administration of estradiol 17β (E_2) to heifers on Day 13 of the cycle initiated luteolysis by increasing the $PGF_{2\alpha}$ concentration (17). We have recently shown that soy bean–derived phytoestrogens regulate both $PGF_{2\alpha}$ and PGE_2 secretion *in vivo* in endometrium during the estrous cycle and early pregnancy in cattle (18, 19). In ruminants, $PGF_{2\alpha}$ is the major luteolytic agent (20), whereas PGE_2 has luteoprotective and anti-luteolytic properties (21, 22). Therefore, achieving an optimal $PGF_{2\alpha}$ to PGE_2 ratio is essential for endometrial receptivity, maintenance of corpus luteum (CL), and P4 secretion (23). It has been demonstrated that endometrial epithelial cells synthesize mainly $PGF_{2\alpha}$, whereas endometrial stromal cells synthesize approximately 10 times more of luteotropic PGE_2 than epithelial cells (22, 24, 25). Therefore, it is important to determine which cells are target cells for their action of crude phytoestrogens and their metabolites in bovine endometrium.

Phytoestrogens have structural similarity to E_2. Therefore, we suppose that they elicit or selectively modulate genomic estrogenic responses by binding to both α and β estrogen receptors (ERs) (26, 27). We also suppose that phytoestrogens elicit or selectively modulate nongenomic estrogenic responses by their influence on protein kinase A action and intracellular calcium (Ca^{2+}) mobilization (28–30).

The aims of the present study were to determine (i) which endometrial cells, epithelial or stromal, are the target of phytoestrogens and their metabolites for PG synthesis, (ii) what is the intracellular mechanism of the phytoestrogen-dependent increase of $PGF_{2\alpha}$ synthesis in endometrial epithelial and stromal cells, and (iii) whether phytoestrogens and E_2 cause intracellular Ca^{2+} mobilization in endometrial epithelial and stromal cells. In the present study, we selected two major soy-derived phytoestrogens (i.e., daidzein, genistein) and their metabolites (i.e., equol and para-ethyl-phenol, respectively), which have been identified in the serum of cows fed diets rich in soy bean (18, 19).

Materials and Methods

Animals and Collection of Endometrial Tissue. Bovine uteri were obtained at a local abattoir within 30 mins of exsanguination and were transported, on ice, to the laboratory within 1 hr. Estimation of the stages of the estrous cycle was determined by macroscopic observation of the ovaries and uteri (31). The uterine horns were separated from each other and from the remaining tissue.

Cell Isolation, Culture, and Experiments. In this study, uteri of the early estrous cycle (i.e., Days 2–5) were used. The epithelial and stromal cells from the bovine endometrium were enzymatically separated (0.05% collagenase; #C0130; Sigma-Aldrich, St. Louis, MO) using procedures previously described (25). Cell viability was higher than 85%. The obtained cells consisted of stromal and epithelial cells with only a few fibroblasts.

The final pellet of both the stromal and epithelial cells was suspended in a culture medium, Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1 [v/v]; #D8900; Sigma-Aldrich) containing 10% calf serum (#16170-078; Gibco BRL, Grand Island, NY) and 20 μg/ml gentamicin (#15750-060; Gibco BRL). The cells of each type were separately seeded at a density of 1×10^5 viable cells/ml in 48-well plates (Experiments 1 and 2; #150687; Costar, Cambridge, MA) or 30-ml bottles (Experiment 3; #83.1813.300; Sarstedt, Numbrecht, Germany) and cultured at 37.5°C in a humidified atmosphere of 5% CO_2 and 95% air. To purify the stromal preparation, the medium was changed 6 hrs after plating, at which time selective attachment of stromal cells had occurred. Alternatively, because the epithelial cells attached 24 hrs to 48 hrs after plating, the medium in the epithelial cell culture was replaced 48 hrs after plating. The medium was changed every 2 days until confluency was reached. When the cells were confluent (6–7 days after the start of the culture), the medium was replaced with fresh DMEM and Ham's F12 medium, supplemented with 0.1% bovine serum albumin (BSA; #A9056; Sigma-Aldrich), 5 ng/ml sodium selenite (#S1382; Sigma), 0.5 m*M* ascorbic acid (#A1417; Sigma-Aldrich), 5 μg/ml transferring, and 20 μg/ml gentamicin (#G-1397; Sigma-Aldrich). In Experiment 3, the cells were trypsinized from the bottles and suspended in calcium-free Hanks' balanced salt solution (HBSS) supplemented with 0.1% BSA. The cells suspended in HBSS were then treated with phytoestrogens for intracellular Ca^{2+} mobilization measurement. The cells from 48-well plates were then exposed to various stimulators for the following experiments.

Experiment 1. To determine the possible differential effect of phytoestrogen on epithelial and stromal cells, each type of cell was exposed to equol (10^{-8} *M*; #45405; Fluka Chemie GmbH, Buch, Switzerland), para-ethyl-phenol (10^{-8} *M*; #821290; Merck & Co., Inc., Gibbtown NJ), daidzein (10^{-8} *M*; #30405; Fluka Chemie GmbH), and genistein (10^{-8} *M*; #345834; Calbiochem-Novabiochem GmbH, Bad Soden, Germany) for 24 hrs. Estradiol 17β (10^{-9} *M*; #75262; Fluka Chemie GmbH) was used as reference compound. Tumor necrosis factor-α (TNF-α; 6×10^{-11} *M*; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) and oxytocin (10^{-6} *M*; #O4375; Sigma-Aldrich) were used as positive controls for stromal and epithelial cells, respectively (25, 31). An enzyme immunoassay measured $PGF_{2\alpha}$ and PGE_2 concentrations in culture medium.

Experiment 2. To determine the intracellular mechanism of phytoestrogen action on the bovine endometrial epithelial and stromal cells, the cells were preincubated for 0.5 hrs with an ER antagonist (ICI-7α,17β-[9[(4,4,5,5,5-

pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; 10^{-6} *M*; #129453-61-8; Tocris Cookson Inc, Ellisville, MO), a translation inhibitor (actinomycin D; 12.5 × 10^{-4} *M*; #114666; Calbiochem-Novabiochem GmbH), a protein kinase A (PKA) inhibitor (staurosporin; 10^{-7} *M*; #569398; Calbiochem, EMD Biosciences, San Diego, CA), and a phospholipase C (PLC) inhibitor (U73122; 10^{-6} *M*; #662035; Calbiochem, EMD Bioscience). After this pre-incubation, the cells were stimulated with equol (10^{-8} *M*), para-ethyl-phenol (10^{-8} *M*), and E_2 as the reference compound (10^{-9} *M*). The two phytoestrogen metabolites are present in high concentrations, in conjugated form, in the blood plasma of cows that are fed diets rich in soy bean (18, 19). Unconjugated, active phytoestrogen metabolites are also present in the blood plasma of these cows in high concentrations. The same concentrations of phytoestrogens found in the plasma were used for treatment in the present *in vitro* study (18, 19). Concentrations of $PGF_{2\alpha}$ in culture medium were measured by an enzyme immunoassay.

After the culture, the conditioned media were collected in tubes with 5 µl EDTA and 1% aspirin solution (pH 7.3; #A209; Sigma-Aldrich) and frozen until measurement of $PGF_{2\alpha}$ and PGE_2. Protein content was estimated by the method of Lowry *et al.* (32) and was used to standardize $PGF_{2\alpha}$ and PGE_2 concentrations. The results were expressed as ng/µg protein.

Experiment 3. To determine the effect of phytoestrogens on intracellular Ca^{2+} mobilization, endometrial epithelial and stromal cells were exposed to equol (10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M*), para-ethyl-phenol (10^{-8} *M*, 10^{-7} *M*, 10^{-6} *M*), and E_2 as the reference compound (10^{-9} *M*, 10^{-8} *M*, and 10^{-7} *M*). The intracellular calcium mobilization was measured by the quantitative method described by Skarzynski and Okuda (33). In the method cell permeable form of the fluorescent Ca^{2+} indicator, Fura-2 (Fura-2 AM; #384-0583; Dojindo, Kumamoto, Japan) was used.

Intracellular Calcium Mobilization. In the quantitative method, epithelial and stromal cells were trypsinized from the bottles and suspended in calcium-free HBSS supplemented with 0.1% BSA. Then, the cells were washed three times by centrifugation in calcium-free HBSS (5 mins; 100 *g*). Fura-2 AM, the lipophilic acetoxymethylester form of Fura-2, was dissolved in dimethyl sulfoxide to form a 1-m*M* stock solution, and 10 µl was added to 2-ml cell suspensions (5 µ*M* final concentration) to preload the cells with dye. The cells were incubated for 30 mins at 37°C and then washed three times in calcium-free HBSS. After washing, the cells were postincubated for 30 mins in HBSS at room temperature to ensure full hydrolysis of the Fura-2 ester. Spectrofluorometric measurements were conducted in 1.5-ml samples continuously stirred in a quartz-glass cuvette and thermostatically maintained at 37°C. Fluorescence was monitored using a Shimadzu spectrofluorometer RF-5000 (Shimazu, Kyoto, Japan). In millisecond intervals, the intensity of fluorescence was measured in the cells treated with equol (10^{-8} *M* in the 60th second of the experiment, 10^{-7} *M* in the 120th second of the experiment, and 10^{-6} *M* in the 180th second of the experiment), para-ethyl-phenol (10^{-8} *M* in the 60th second of the experiment, 10^{-7} *M* in the 120th second of the experiment, and 10^{-6} *M* in the 180th second of the experiment), and E_2 (10^{-9} *M* in the 60th second of the experiment, 10^{-8} *M* in the 120th second of the experiment, and 10^{-7} *M* in the 180th second of the experiment). Excitation and emission wavelengths were 340 nm and 490 nm, respectively, with slit widths of 5 nm for both wavelengths. Intracellular $[Ca^{2+}]_i$ concentrations were calculated from the following equation:

$$[Ca^{2+}]_i = KD \times [(F - F_{min})/(F_{max} - F)],$$

where F is the fluorescence in the examined sample and KD is the dissociation constant for Fura-2–Ca^{2+} complex at 37.5°C is 2.24×10^{-7} *M*. Maximum fluorescence (F_{max}) was measured by maximum mobilization of the Fura-2–Ca^{2+} complex with phorbol 12-myristate 13-acetate (10^{-7} *M*; #P148; Sigma-Aldrich).

Hormone Determination. Concentrations of PGE_2 and $PGF_{2\alpha}$ in the culture medium were determined with the enzyme immunoassays as previously described (31, 33). The PGE_2 standard curve ranged from 0.39 ng/ml to 100 ng/ml, and the ED_{50} of the assay was 6.25 ng/ml. The intra- and interassay coefficients of variation were 1.6% (n = 10) and 11.0% (n = 10), respectively. The $PGF_{2\alpha}$ standard curve ranged from 0.016 ng/ml to 4 ng/ml, and the ED_{50} of the assay was 0.25 ng/ml. The intra- and interassay coefficients of variation were, on average, 11.3% (n = 10) and 7.1% (n = 10), respectively.

Statistical Analysis. The data obtained from the Experiments 1 and 2 are shown as the mean ± SEM of values obtained in four separate experiments, each performed in triplicate. The statistical significance of differences between control and treated groups was assessed by one-way ANOVA followed by Bonferroni's multiple comparison test. Patterns of Ca^{2+} mobilization in Experiment 3 were estimated by tests for repeated measures. All tests were performed by computer using Prism 4 software (GraphPad PRISM; GraphPad Software, Inc., San Diego, CA).

Results

Effects of Oxytocin, Phytoestrogens, Their Metabolites, and E_2 on $PGF_{2\alpha}$ and PGE_2 Production in Epithelial Cells. Figure 1 shows $PGF_{2\alpha}$ (a) and PGE_2 (b) production by epithelial cells in response to oxytocin, equol, para-ethyl-phenol, daidzein, genistein, and E_2. Equol, para-ethyl-phenol, daidzein, and genistein (all 10^{-8} *M*) stimulated the secretion of $PGF_{2\alpha}$ in epithelial cells (5.6-, 5.8-, 6.2-, and 6.0-fold, respectively; $P < 0.001$) compared with controls. Equol, para-ethyl-phenol, daidzein, and genistein (all 10^{-8} *M*) stimulated the secretion of PGE_2 in epithelial cells (3.0-, 2.3-, 3.4-, and 3.7-fold, respectively; $P < 0.001$). At a concentration of 10^{-9} *M*, E_2 stimulated $PGF_{2\alpha}$ and PGE_2 secretion in

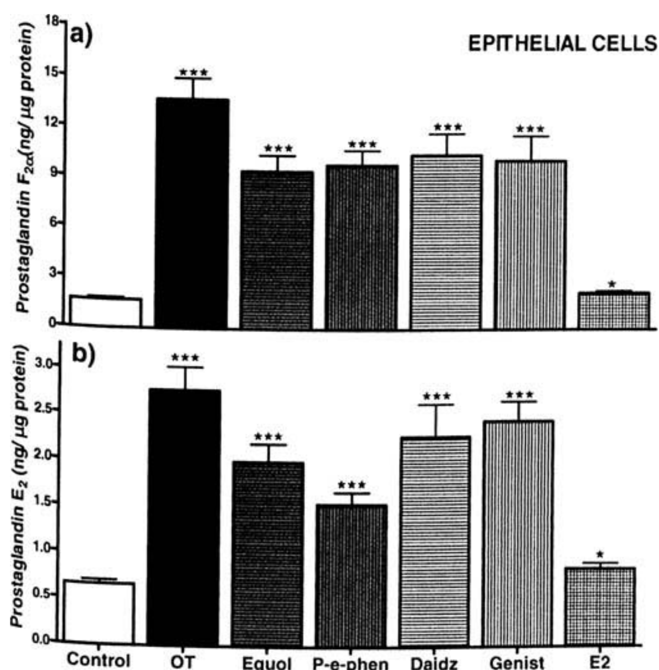

Figure 1. The effects of oxytocin (OT), equol, para-ethyl-phenol (P-e-phen), daidzein (Daidz), and genistein (Genist) on the production of prostaglandin (PG) $PGF_{2\alpha}$ (a) and PGE_2 (b) by bovine epithelial cells. Oxytocin (10^{-6} *M*), phytoestrogens, and their metabolites (all 10^{-8} *M*) were added 24 hrs before the end of culture. E2, estradiol 17β. Asterisks indicate significant differences between control and treated groups (* $P < 0.05$, *** $P < 0.001$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test ($n = 4$).

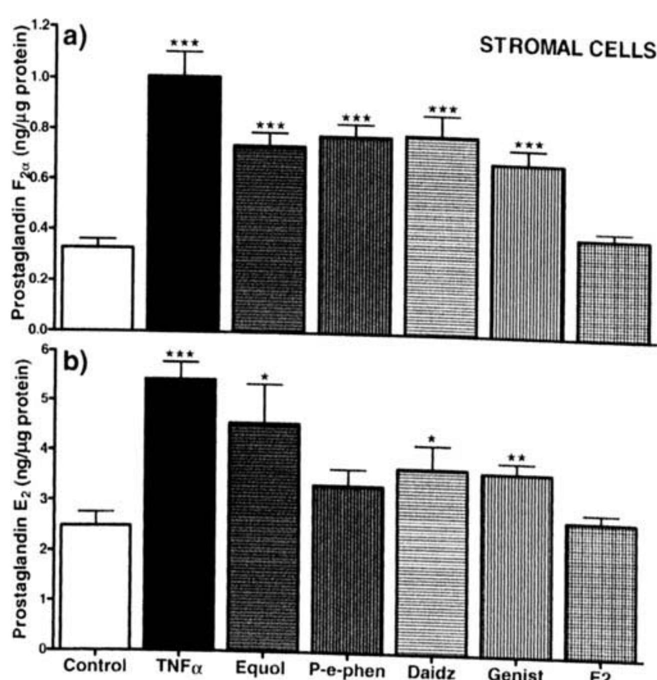

Figure 2. The effects of tumor necrosis factor-α, equol, para-ethyl-phenol, daidzein, and genistein on the production of prostaglandin (PG) $PGF_{2\alpha}$ (a) and PGE_2 (b) by bovine stromal cells. Tumor necrosis factor-α (TNFα; 6×10^{-11} *M*), phytoestrogens, and their metabolites (all 10^{-8} *M*) were added 24 hrs before the end of culture. Asterisks indicate significant differences between control and treated groups (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test ($n = 4$).

epithelial cells ($P < 0.05$). Oxytocin, at a concentration of 10^{-7} *M*, stimulated the production of both PGs in epithelial cells ($P < 0.001$), which accounts for appropriate responsiveness of the cells.

Effects of TNF-α, Phytoestrogens, Their Metabolites, and E_2 on $PGF_{2\alpha}$ and PGE_2 Production in Stromal Cells. Figure 2 shows $PGF_{2\alpha}$ (a) and PGE_2 (b) production by stromal cells in response to TNF-α, equol, para-ethyl-phenol, daidzein, genistein, and E_2. Equol, para-ethyl-phenol, daidzein, and genistein (all 10^{-8} *M*) stimulated the secretion of $PGF_{2\alpha}$ in stromal cells (2.3-, 2.4-, 2.4-, and 2.1-fold, respectively; $P < 0.001$). Equol, daidzein, and genistein (all 10^{-8} *M*) stimulated the secretion of PGE_2 in stromal cells (1.8-, 1.5-, and 1.4-fold, respectively; $P < 0.001$). At a concentration of 6×10^{-11} *M*, TNF-α stimulated the production of both PGs in stromal cells ($P < 0.001$), which accounts for appropriate responsiveness of the cells.

Effects of Phytoestrogens on the $PGF_{2\alpha}$ to PGE_2 Ratio in Epithelial and Stromal Cells. The basal $PGF_{2\alpha}$ to PGE_2 ratio in untreated cells (control) was 2.6 ± 0.2 and 0.2 ± 0.02 in epithelial and stromal cells, respectively. Treatment with phytoestrogens and their

Table 1. The Effects of Oxytocin, Tumor Necrosis Factor-α, Phytoestrogens, and Their Metabolites on the $PGF_{2\alpha}$ to PGE_2 Ratio in Epithelial and Stromal Cells

	$PGF_{2\alpha}$ to PGE_2 ratio	
Treatment	Epithelial cells	Stromal cells
Control	2.6 ± 0.2[a]	0.2 ± 0.02[b]
Oxytocin	6.0 ± 0.9*	N/A
Tumor necrosis factor-α	N/A	0.2 ± 0.02[b]
Equol	5.0 ± 0.4[a]*	0.2 ± 0.02[b]
Para-ethyl-phenol	7.4 ± 1.5[a]**	0.2 ± 0.02[b]
Daidzein	5.7 ± 0.8[a]*	0.3 ± 0.02[b]
Genistein	5.0 ± 1.1[a]*	0.2 ± 0.02[b]

[a,b] Different superscript letters indicate significant differences ($P < 0.05$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Asterisks indicate significant differences compared with control as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGE_2, prostaglandin estradiol 17β; N/A, not applicable.

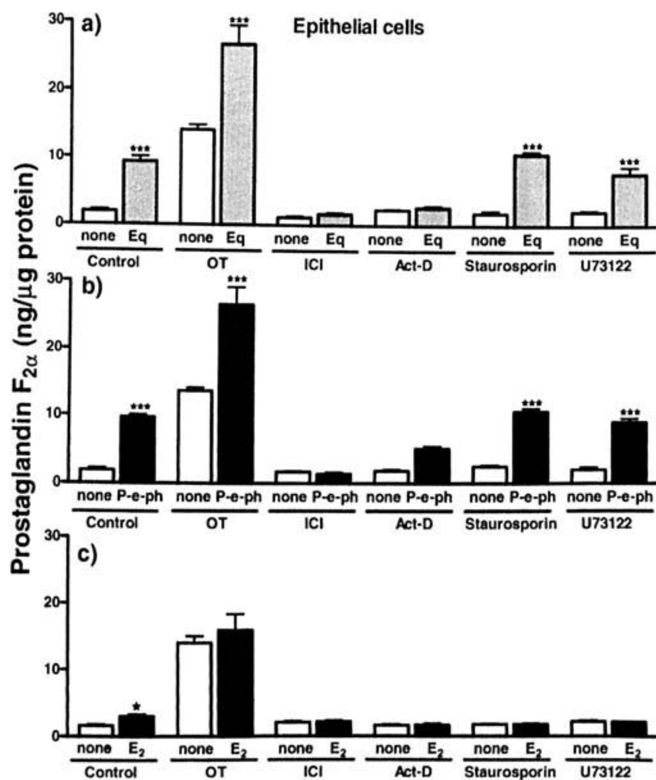

Figure 3. The influence of an estrogen-receptor antagonist (i.e., ICI), a translation inhibitor (i.e., actinomycin D [Act-D]), a protein kinase A inhibitor (i.e., staurosporin), and a phospholipase C inhibitor (i.e., U73122) on equol-stimulated (a) and para-ethyl-phenol–stimulated (b) prostaglandin (PG) $PGF_{2\alpha}$ production in bovine epithelial cells. The cells were preincubated for 0.5 hrs with ICI (10^{-6} *M*), actinomycin D (12.5×10^{-4} *M*), staurosporin (10^{-7} *M*), and U73122 (10^{-6} *M*) and then stimulated with equol (10^{-8} *M*) and para-ethyl-phenol (10^{-8} *M*). Asterisks indicate significant differences between control and treated groups (* $P < 0.05$, *** $P < 0.001$) as determined by two-way ANOVA followed by Bonferroni's multiple comparison test.

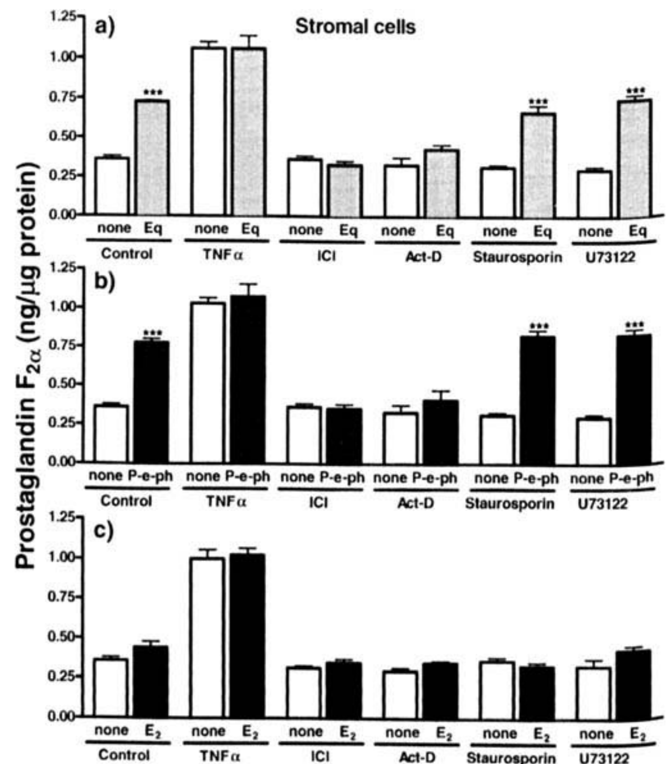

Figure 4. The influence of an estrogen-receptor antagonist (i.e., ICI), a translation inhibitor (i.e., actinomycin D), a protein kinase A inhibitor (i.e., staurosporin), and a phospholipase C inhibitor (i.e., U73122) on equol-mediated (a) and para-ethyl-phenol–mediated (b) prostaglandin (PG) $PGF_{2\alpha}$ production in bovine stromal cells. The cells were preincubated for 0.5 hrs with ICI (10^{-6} *M*), actinomycin D (12.5×10^{-4} *M*), staurosporin (10^{-7} *M*), and U73122 (10^{-6} *M*) and then stimulated with equol (10^{-8} *M*) and para-ethyl-phenol (10^{-8} *M*). Asterisks indicate significant differences between control and treated groups (*** $P < 0.001$) as determined by two-way ANOVA followed by Bonferroni's multiple comparison test.

metabolites induced an increase in the $PGF_{2\alpha}$ to PGE_2 ratio in epithelial cells, but it did not affect the $PGF_{2\alpha}$ to PGE_2 ratio in stromal cells (Table 1). In epithelial cells, phytoestrogens and their metabolites significantly increased the $PGF_{2\alpha}$ to PGE_2 ratio from 2.6 ± 0.2 (control) to 5.7 ± 0.8 (daidzein), 5.0 ± 1.1 (genistein), 5.0 ± 0.4 (equol), and 7.4 ± 1.5 (para-ethyl-phenol), respectively ($P < 0.05$).

Effects of ICI, Actinomycin D, Staurosporin, and U73122 on Phytoestrogen and E_2-Stimulated $PGF_{2\alpha}$ Production in Epithelial and Stromal Cells. The PKA inhibitor (staurosporin) and PLC inhibitor (U73122) did not affect the stimulative effect of active phytoestrogen metabolites equol (a) and para-ethyl-phenol (b) on $PGF_{2\alpha}$ production by epithelial (Fig. 3) and stromal (Fig. 4) cells ($P > 0.05$). The stimulative effect of examined substances on $PGF_{2\alpha}$ production by epithelial and stromal cells was reduced by the ER antagonist, ICI, and the translation inhibitor, actinomycin D ($P < 0.001$). The stimulative effect of E_2 on $PGF_{2\alpha}$ production by epithelial cells was reduced by all antagonists and inhibitors used ($P < 0.05$).

Effects of Phorbol 12-Myristate 13-Acetate, Phytoestrogen Metabolites, and E_2 on Intracellular Ca^{2+} Mobilization in Epithelial and Stromal Cells. Neither equol nor para-ethyl-phenol (10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M* for both) caused the intracellular Ca^{2+} mobilization in epithelial and stromal cells (Fig. 5a and b; $P > 0.05$). Estradiol 17β (10^{-9} *M*, 10^{-8} *M*, and 10^{-7} *M*) caused the intracellular Ca^{2+} mobilization in epithelial and stromal cells (Fig. 5c; $P < 0.05$). Phorbol 12-myristate 13-acetate (10^{-7} *M*) induced the intracellular Ca^{2+} mobilization in epithelial and stromal cells (Fig. 5a and b; $P < 0.05$), which accounts for appropriate responsiveness of the cells.

Discussion

The present study demonstrated that, in bovine endometrium, phytoestrogens and their metabolites mainly stimulated $PGF_{2\alpha}$ production in epithelial cells. Moreover, our results indicated that phytoestrogens and their active metabolites act mainly through a genomic, ER-dependent mechanism without intracellular calcium mobilization.

The main source of $PGF_{2\alpha}$ in bovine endometrium is known to be epithelial cells, whereas stromal cells produce

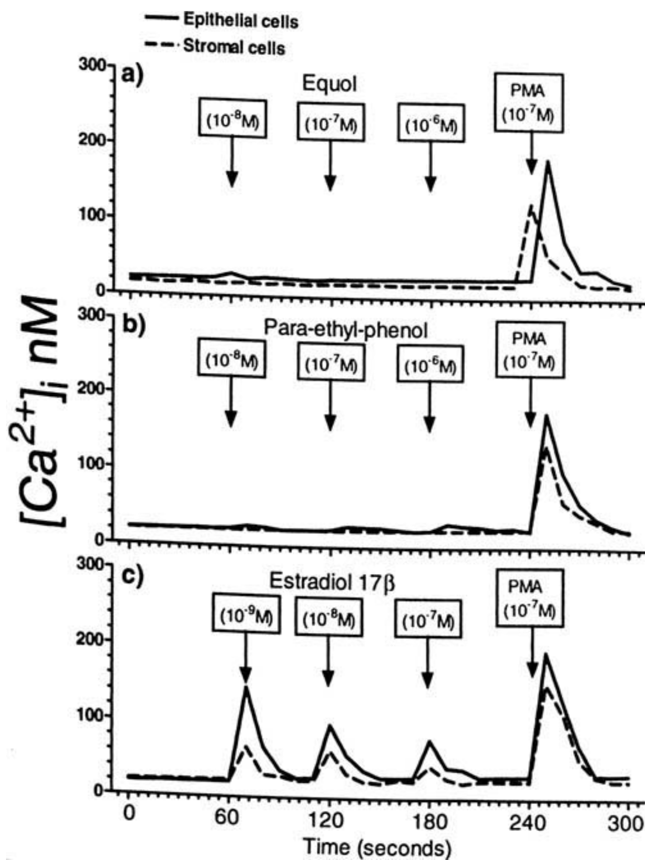

Figure 5. The influence of equol (10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M*) (a) and para-ethyl-phenol (10^{-8} *M*, 10^{-7} *M*, and 10^{-6} *M*) (b) on the concentrations of intracellular calcium in epithelial cells (straight line) and stromal cells (dotted line) of bovine endometrium. Changes of calcium concentrations are shown as changes in the fluorescence of Ca^{2+} (Fura-2 AM) complex. Phorbol 12-myristate 13-acetate (10^{-7} *M*) was used as a positive control.

approximately 10 times more of luteotropic PGE_2 in comparison to epithelial cells (22, 24, 25). In the present study, phytoestrogens and their metabolites preferentially stimulated $PGF_{2\alpha}$ synthesis in epithelial cells (560%–620% of control) compared with stromal cells (210%–240% of control) and moderately, but significantly, increased PGE_2 production in both epithelial cells (230%–370%) and stromal cells (140%–180%). In comparison, E_2 stimulated $PGF_{2\alpha}$ and PGE_2 production (140% for both PGs) only in epithelial cells ($P < 0.05$). Our data showed that the basal $PGF_{2\alpha}$ to PGE_2 ratio in epithelial cells was 10 times higher than the $PGF_{2\alpha}$ to PGE_2 ratio in stromal cells. Moreover, treatment with phytoestrogens and their metabolites resulted in a 2.5 to 3.5 time increase in the $PGF_{2\alpha}$ to PGE_2 ratio in epithelial cells. In contrast, the same treatment with phytoestrogens did not modify the $PGF_{2\alpha}$ to PGE_2 ratio in stromal cells. Moreover, phytoestrogen metabolites have an enhancing effect on $PGF_{2\alpha}$ production in oxytocin-treated epithelial cells. This synergic effect of phytoestrogens and oxytocin may additionally increase the ratio of luteolytic $PGF_{2\alpha}$ to luteotropic PGE_2 production in bovine endometrium. The results indicate that phytoestrogens and their metabolites differentially modulate PG synthesis in a cell-specific manner, increasing both PGs without altering the $PGF_{2\alpha}$ to PGE_2 ratio in stromal cells and directing the biosynthetic pathway toward $PGF_{2\alpha}$ in epithelial cells. Thus, it appears that phytoestrogens and their metabolites mainly modulate the $PGF_{2\alpha}$ to PGE_2 ratio in epithelial cells. In the endometrium, a proper $PGF_{2\alpha}$ to PGE_2 ratio is essential for maintaining an optimum uterine environment for embryo implantation and development (21, 34). During embryo development and implantation, the $PGF_{2\alpha}$ to PGE_2 ratio decreases due to an increase of PGE_2 production (34). The increased PGE_2 stimulates P4 synthesis in the confidence limits (35). Based on the stronger phytoestrogen-dependent stimulation of $PGF_{2\alpha}$ compared with PGE_2 production in epithelial cells observed in the present study, we assume that phytoestrogens cause premature luteolysis, leading to embryonic loss during early pregnancy in cattle. This supposition has been supported by our recent *in vivo* and *in vitro* findings (19). We have shown that soy bean–derived phytoestrogens and their metabolites act as endocrine disruptors, leading to disruption of the reproductive processes and temporal infertility of cows. Phytoestrogens and their active metabolites disrupt the ratio of PGE_2 to $PGF_{2\alpha}$, which leads to the nonphysiologic production of luteolytic agent in cattle during the estrous cycle and pregnancy (19).

Estrogens exert their physiologic effects in target cells by genomic (36) and nongenomic pathways (37), as shown by the results obtained in Experiment 2. The genomic pathway involves activation of ERs and modification of gene expression (38–41). The genomic mechanism of estrogen action depends on the presence of α and β, two types of ER (42). It has been proven that some phytoestrogens have higher affinity to ERβ than to ERα (8, 43). Phytoestrogens and their active metabolites may compete with endogenous E_2, thus disturbing the processes influenced by E_2. The stimulatory effect of E_2 on the synthesis of both PGs in epithelial cells was reduced by an ER antagonist (ICI) and a translation inhibitor (actinomycin D) supporting its genomic action. In the present study, ICI and actinomycin D also blocked the stimulatory effect of equol and para-ethyl-phenol on $PGF_{2\alpha}$ synthesis in endometrial epithelial and stromal cells. The study of Dubey *et al.* (14) revealed that a selective ER antagonist, ICI, blocked the E_2 influence on endothelial cells of the blood vessels, suggesting genomic action mediated by ERα. The results of Wang *et al.* (26), and Wang and Kurzer (27) proved that phytoestrogens act *via* binding to both ERα and β. Therefore, we assume that, owing to phytoestrogen structural similarity to E_2, they may elicit or selectively modulate genomic estrogenic responses by binding to both ERs, like endogenous E_2.

In the nongenomic pathway of estrogen action, PLC and PKA are the most important compounds of the intracellular second messenger system. The results of the present study contrasted with findings of some research

groups and agreed with others. Morley *et al.* (28), Katzenellenbogen (29), and Smith (30) found that endogenous E_2 can act *via* a nongenomic way, especially *via* PKA action and intracellular calcium (Ca^{2+}) mobilization. Szego (44) found that endogenous steroids induced signaling pathways connected with membrane-bound enzymes such as PLC and PKA, which lead to the intracellular increase of cAMP and calcium mobilization (45, 46). In our study, the stimulatory effect of E_2 on the synthesis of both PGs in epithelial cells was reduced by a PLC inhibitor, U73122, and a PKA inhibitor, staurosporin, supporting its nongenomic action. On the other hand, Dubey *et al.* (14) found that genistein inhibited mitogen-activated protein kinase activity and mRNA for transforming growth factor–β and integrin $\alpha_v\beta_3$ expression (47), tyrosine kinase activity (48), phospholipase D activity (49), and PLC-dependent intracellular calcium release (50). However, in the present study, neither the PLC inhibitor nor the PKA inhibitor (inhibitors of nongenomic pathways and second messengers) inhibited equol-mediated and para-ethyl-phenol–mediated stimulation of $PGF_{2\alpha}$ synthesis in epithelial and stromal cells. These results suggest the lack of a nongenomic mechanism of phytoestrogen metabolites action on the PG synthesis in bovine endometrium, in contrast to endogenous E_2.

In conclusion, the present study demonstrated that phytoestrogens stimulate both $PGF_{2\alpha}$ and PGE_2 in both cell types of bovine endometrium *via* an ER-dependent genomic pathway. However, because phytoestrogens preferentially stimulated $PGF_{2\alpha}$ synthesis in epithelial cells of bovine endometrium, they may disrupt uterus function by altering the $PGF_{2\alpha}$ to PGE_2 ratio. This action of phytoestrogens on $PGF_{2\alpha}$ may account, at least in part, for the reproductive disorders observed in ruminants fed diets that are rich in soy.

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