BIOLOGY OF REPRODUCTION **68,** 1430–1436 (2003)
Published online before print 27 November 2002.
DOI 10.1095/biolreprod.102.006569

Differential Effects of Natural and Environmental Estrogens on Endothelin Synthesis in Bovine Oviduct Cells[1]

Karin C. Reinhart,[3] Raghvendra K. Dubey,[3,4] Barbara Cometti,[3] Paul J. Keller,[3] and Marinella Rosselli[2,3]

Department of Obstetrics and Gynecology,[3] Clinic for Endocrinology, University Hospital, 8091 Zurich, Switzerland
Department of Medicine,[4] Center for Clinical Pharmacology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15224

ABSTRACT

Endothelin-1 (ET-1), a vasoconstrictor and mitogenic peptide that plays an important role within the endocrine/reproductive system, is synthesized by oviduct cells and regulates tubal contractility. Because 17β-estradiol (estradiol) regulates oviduct function by influencing the synthesis of autocrine/paracrine factors, estradiol may also regulate ET-1 synthesis. Furthermore, environmental estrogens (EEs; phytoestrogens and xenoestrogens), which structurally resemble estradiol and possess estrogenic activity, may mimic the effects of estradiol on ET-1 synthesis and may influence the reproductive system. Using cultures of bovine oviduct cells (epithelial cells:fibroblasts, 1:1), we investigated and compared the modulatory effects of estradiol, phytoestrogens, and xenoestrogens on ET-1 synthesis and determined whether these effects were estrogen receptor (ER) mediated. A quantitative ELISA for ET-1 in the culture medium revealed that 17β-estradiol inhibits ET-1 synthesis in a concentration-dependent manner (4–400 nmol/L). In contrast to estradiol, ET-1 synthesis was induced in cell cultures treated with xenoestrogens in the following order of potency (0.1 μmol/L): 4-hydroxy-trichlorobiphenyl > 4-hydroxy-dichlorobiphenyl > trichlorobiphenyl. The stimulatory effects of xenoestrogens on ET-1 production were mimicked by the phytoestrogens biochanin-A and genistein but not by formononetin, equol, and daidzein. The oviduct cells expressed both ERs (α and β), but the modulatory effects of estradiol, but not EEs, on ET-1 synthesis were blocked by ICI-182 780 (1 μM), a pure ER antagonist. Our results provide evidence that estradiol inhibits ET-1 synthesis in oviduct cells via an ER-dependent mechanism, whereas, EEs induce ET-1 synthesis via an ER-independent mechanism. The contrasting effects of EEs on ET-1 synthesis suggests that EEs may act as endocrine modulators/disruptors and may have deleterious effects on the reproductive system by adversely influencing the biology and physiology of the oviduct.

environment, estradiol receptor, growth factors, oviduct, toxicology

INTRODUCTION

The oviduct plays a major role in the reproductive process by regulating the transport of the gametes and embryo(s) and providing a positive microenvironment for the fertilization process and the primary stages of embryo development [1]. The rhythmic/cyclic contraction and relaxation of smooth muscle cells and the coordinated ciliary beats of the oviduct cells play a critical role in the transport of the gametes and embryo(s), a process regulated by multiple autocrine-paracrine factors synthesized within the oviduct wall. Oviduct cells synthesize dilatory factors such as nitric oxide [2] and prostaglandins [3] and constricting factors such as thromboxane [3] and endothelin [4]. Because most dilatory factors and constricting factors are growth inhibitors or growth inducers [5], these factors also may play a key role in regulating the early phases of embryo development and in facilitating the implantation process.

The oviduct physiology is regulated by ovarian hormones such as 17β-estradiol, which indirectly influences oviduct function and the fertilization process by controlling the synthesis of autocrine/paracrine factors within the oviduct [6]. Estradiol induces the synthesis of leukemia inhibitory factor [7], which plays a key role in implantation. Endothelin (ET-1), a potent contracting factor, is synthesized by cultured bovine oviduct epithelial cells and is able to induce contraction of bovine oviduct segments [4, 8].

The biologic effects of estradiol are largely mediated via estrogen receptors (ERα and ERβ); however, emerging evidence also supports nongenomic and non-receptor-mediated estradiol effects [9]. The endocrine effects of estradiol can be mimicked and blocked by several chemical agents that are structurally similar to estradiol, agents that bind to ERs or posses estrogenic activity. These estrogen like chemicals are termed environmental estrogens (EEs) and are classified into two major categories: phytoestrogens (plant-derived estrogens) and xenoestrogens (man-made estrogenic chemicals).

Environmental estrogens interfere with the reproductive process of humans and other species [9]. Although the deleterious effects of environmental estrogens on the reproductive system have been well established, the mechanisms involved remain undefined. The fact that some environmental estrogens induce estrogenic effects whereas others are antiestrogenic [9] has further complicated the issue. Moreover, recent studies have provided evidence that compared with estradiol many of the environmental estrogens known to act as endocrine disruptors have a very low binding affinity for ERα and ERβ [9], suggesting that they may mediate their effects via alternative ER-independent mechanisms.

The aims of the present study were to determine whether 1) ET-1 synthesis by oviduct cells is regulated by estradiol, 2) phytoestrogens and xenoestrogens influence the synthesis of ET by oviduct cells, 3) oviduct cells express ERα and ERβ, and 4) the effects of estradiol, phytoestrogens, and polychlorinated biphenyls (PCBs) on ET-1 synthesis are ER mediated. For the present study, we selected five

[1]This work was supported by the Swiss National Science Foundation (grants 32-55738.98 and 32-64040.00).
[2]Correspondence. FAX: 41 1 255 44 39;
e-mail: marinella.rosselli@fhk.usz.ch

Received: 17 April 2002.
First decision: 28 May 2002.
Accepted: 1 November 2002.

ISSN: 0006-3363. http://www.biolreprod.org

major soya-derived phytoestrogens (genistein, daidzein, biochanin A, equol, and formononetin), one PCB (trichlorobiphenyl [TCB]), and two hydroxylated PCBs (4-hydroxy-dichlorobiphenyl [4-OH-DCB] and 4-hydroxy-trichlorobiphenyl [4-OH-TCB]), which have been identified in human and animal sera.

MATERIALS AND METHODS

Isolation and Culture of Bovine Oviduct Cells

Oviducts of young cyclic nonpregnant cows were obtained from the local abattoir, and oviduct cells (mixed population of epithelial cells and fibroblasts, 1:1 ratio) were cultured in Ham F10 (Sigma, Buchs, Switzerland) containing 10% fetal calf serum (FCS, steroid-free, one batch; Sigma) according to our previously published method [7]. Confluent monolayers of these mixed oviduct cells after 6–8 days in culture were used. These mixed cell cultures were characterized immunohistochemically as previously described [4, 7]. Monoclonal antibodies to epithelial cell cytokeratin (anticytokeratin AE1/AE3; DAKO Diagnostiks AG, Zug, Switzerland) and antibodies against fibroblast vimentin (antivimentin VIM 3B4; DAKO) were used to identify the epithelial cells and fibroblasts in culture. Peroxidase-antiperoxidase staining (DAKO) was used to visualize the antibody reactions.

ET-1 synthesis was investigated in primary cell cultures or cells in first passage. Oviduct epithelial cells synthesize ET-1 [4], and preliminary studies conducted with cultured oviduct fibroblasts demonstrated that these cells also synthesize ET-1 (117 ± 24 pg/mg protein after 4 days; $P < 0.05$ vs. Day 0). Because autocrine/paracrine factors generated by both epithelial cells and fibroblasts may regulate the physiology and biology of the oviduct, we used the coculture system to analyze the effects of natural and environmental estrogens on ET-1 synthesis. Using the same experimental model, we previously demonstrated that estradiol and EEs modulate the synthesis of leukemia inhibitory factor [7], and the effects were similar in epithelial cells, fibroblasts, and cocultured cell systems [10].

Treatment Protocols for ET-1 Synthesis

To study the effects of ovarian hormones on ET-1 synthesis in bovine oviduct cells, confluent monolayers of mixed cell cultures (epithelial cells and fibroblasts, 1:1) were washed twice with Hanks balanced salt solution and treated for 4 days with Dulbecco modified Eagle medium (DMEM)/Ham F12 medium (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 1% FCS (steroid free) and containing or lacking 17β-estradiol (4, 40, 200, and 400 nM; Sigma). To evaluate whether the effects of 17β-estradiol were ER mediated, cells were treated with 17β-estradiol (0.2 μM) or genistein (0.2 μM; Extrasynthèse, Genay, France), an ERβ ligand, with or without ICI 182,780 (1 μM; Tocris, Cookson Ltd., Bristol, U.K.), a specific ER antagonist. To investigate the influence of phytoestrogens and xenoestrogens on ET-1 synthesis, cells were incubated for 4 days with DMEM/Ham F12 medium with 1% FCS (steroid free) with or without various phytoestrogens (0.2–2 μM biochanin A, daidzein, equol, formononetin, and genistein; Extrasynthèse), or xenoestrogens (0.1–10 μM TCB, 4-OH-DCB, and 4-OH-TCB; AccuStandard, New Haven, CT). To evaluate whether the effects of EEs on ET-1 synthesis were ER mediated, the mixed cell cultures were treated for 4 days with TCB, 4-OH-TCB, and 4-OH-DCB (0.1 μM) with or without ICI 182,780 (1 μM).

Quantitative Analysis of ET-1

To analyze ET-1, the cell culture medium/supernatant was collected and microfuged. ET-1 levels in 100-μl aliquots of the supernatant were quantified using a highly sensitive ET-1 human ELISA kit (Biotrak; Amersham, Dubendorf, Switzerland), with the following specification: ET-1 sensitivity of 0.1 pg/ml; cross-reactivity with ET-2 (synthetic) of <1%, cross-reactivity with ET-3 (synthetic) of <0.001%, and very low cross-reactivity with big ET-1 (human and porcine) and sarafotoxin S6b. The inter- and intra-assay coefficients of variation were <10%. To verify that cytoplasmic proteins did not interfere with the assay, standard curves of ET-1 were run in culture medium with or without cytoplasmic extracts of oviduct cells (1 mg/ml; extracted from confluent monolayers of cells grown in 75-cm^2 flasks) and spiked with 0–20 pg/ml of ET-1. The standard curves for ET-1 with or without cytoplasmic extracts and after subtracting the blanks did not vary significantly; the assay curves were linear and almost identical (Fig. 1). The concentrations of ET-1 were calculated using a standard curve run under identical conditions. ET-1 concentrations were normalized to total cell proteins and are presented as picograms per milligram protein. Confluent monolayers were used to rule out the contribution of change in cell number. Total cellular protein levels also were measured.

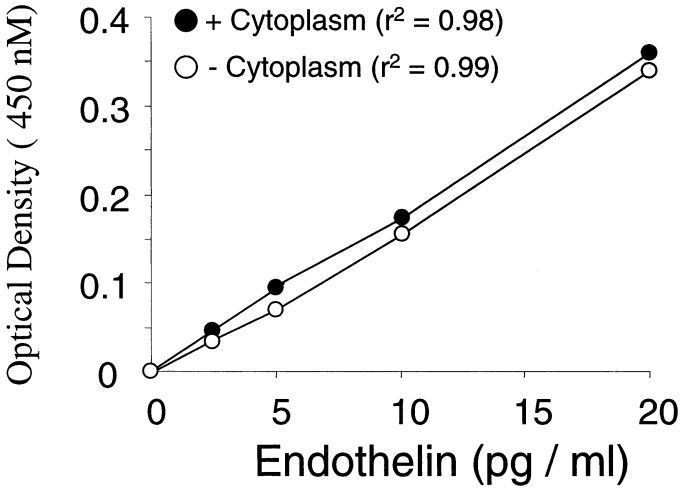

FIG. 1. Comparison of standard curves of ET-1 in medium containing or lacking cytoplasm (1 mg/ml) from mixed cultures of ovarian cells spiked with known concentrations of ET-1. The values for each data point were subtracted with the respective blanks. Cytosolic proteins did not influence the linearity of the ET-1 standard curve, and this curve was parallel to the standard curve obtained with medium spiked with ET-1. The r^2 values obtained from the linear regression of the two curves were 0.98 and 0.99 for the curves with and without cytoplasm, respectively.

Protein Estimation

Cells remaining after the removal of the cell culture supernatant were solubilized in 0.1% SDS, and the total protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad, Glattbrugg, Switzerland) using BSA as the standard. Each experiment was conducted in triplicate and repeated three or four times using cell cultures form different pools of fresh oviducts.

ER Expression and Binding Studies

To investigate whether the oviduct cells express ERα and ERβ, cell lysates from cultured oviduct cells were analyzed by Western blots and probed with antibodies to ERα (purified antiserum to human ERα; Alexis Corp., Lausen, Switzerland) and ERβ (purified antiserum to human ERβ; Alexis). Samples containing 25 μg protein were loaded on 10% SDS-polyacrylamide gels, and proteins were separated by electrophoresis. The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell GmbH, Dassel, Germany) and blocked overnight with 5% milk proteins and 0.2% Tween-30 in PBS. The membranes were probed with antibodies to ERα (dilution 1:1000 in 1% milk protein and 0.2% Tween-20 in PBS) or ERβ (dilution 1:1000) for 1 h. Subsequently, the membranes were washed and treated with a peroxidase-conjugated second antibody (goat anti-rabbit; Pierce, Lausanne, Switzerland). Membranes were then exposed to the substrate (Supersignal west Dura substrate; Pierce), and the labeled proteins were detected by exposing the x-ray films to the membranes.

Receptor binding studies were conducted to ascertain the binding affinity of estradiol to the oviduct cells and to obtain a quantitative estimate for the presence of ERs in oviduct cells. Confluent monolayers of oviduct cells in 35-mm^2 culture dishes were treated with 0.5–8 × 10^{-9} M [^{3}H]17β-estradiol for 1 h at 37°C in serum-free medium. The labeled cytosol was subsequently extracted, and the free estrogen was removed by incubating the cytosol with dextran-coated charcoal. Total binding of [^{3}H]17β-estradiol was quantified by measuring the radioactivity in a liquid scintillation counter, as previously described [10]. Nonspecific binding was measured by parallel incubations in the presence of a 1000-fold excess of unlabeled 17β-estradiol. Binding data was analyzed by nonlinear isotherm equation and by Scatchard analysis (GraphPad).

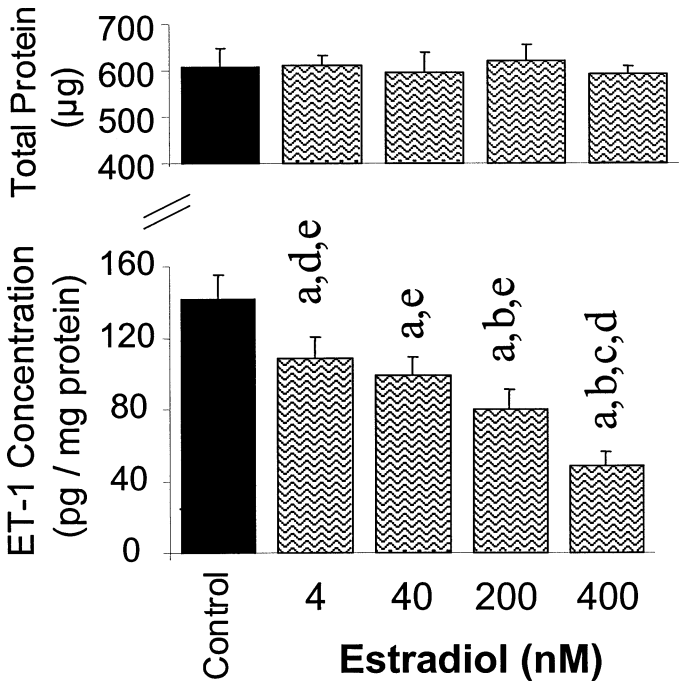

FIG. 2. Concentration-response curves for the effects of 17β-estradiol (4–400 nM) on the synthesis of ET-1 by confluent monolayers of bovine oviduct cell cultures (primary cell culture epithelial cells and fibroblasts, 1:1 ratio) cultured for 4 days with or without (control) 17β-estradiol (bottom). Data (mean ± SD; n = 3 experiments, each in triplicate) were normalized to total protein concentration. The total protein content in controls and cells treated with 4, 40, 200, and 400 nM estradiol did not differ and was 608 ± 40, 611 ± 22, 596 ± 44, 621 ± 35, and 593 ± 17 μg, respectively (top). Letters indicate significant differences ($P < 0.05$) using Student-Newman-Keuls posteriori test: a, $P < 0.05$ vs. control; b, $P < 0.05$ vs. 4 nM estradiol; c, $P < 0.05$ vs. 40 nM estradiol; d, $P < 0.05$ vs. 200 nM estradiol; e, $P < 0.05$ vs. 400 nM estradiol. Similar significance values were obtained by using the Fisher PLSD test. For the ANOVA, F = 29.769 with 4 (treatments) and 10 (residual) degrees of freedom, $P < 0.0001$; lambda = 119.077.

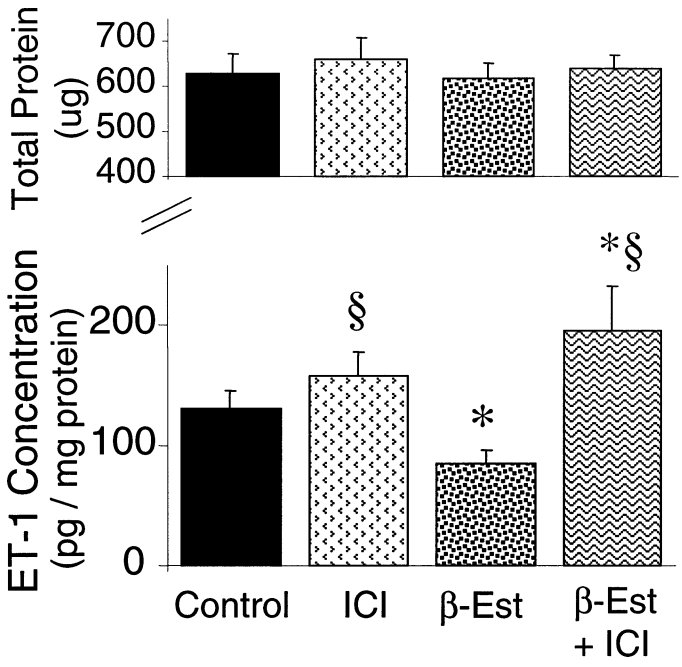

FIG. 3. Modulatory effect of ICI 182,780, a selective ER antagonist, on 17β-estradiol-regulated ET-1 synthesis by confluent monolayers of primary bovine oviduct mixed cell cultures treated for 4 days with 0.2 μM 17β-estradiol (β-Est) with or without ICI 182,780 (1 μM; ICI; bottom). 17β-Estradiol (0.2 μM) significantly inhibited ET-1 synthesis, and ICI 182,780 completely reversed its inhibitory effect. Data represent mean ± SD from three individual experiments, conducted each in triplicate. The amount of ET-1 was normalized to total protein concentration. The total protein content (micrograms) in controls and cells treated with ICI, estradiol, and estradiol plus ICI did not differ and was 629 ± 44, 650 ± 47, 620 ± 33, and 640 ± 29 μg, respectively (top). Symbols indicate significant differences ($P < 0.05$) using Student-Newman-Keuls posteriori test: *$P < 0.05$ vs. control; §$P < 0.05$ vs. estradiol alone. Similar significance values were obtained by using the Fisher PLSD test. For the ANOVA, F = 20.307 with 3 (treatments) and 16 (residual) degrees of freedom, $P < 0.0001$; lambda = 60.922.

Statistical Analysis

Data are presented as mean ± SD. Statistical analysis by ANOVA was performed using the Statview program. For individual comparisons for significant differences, both the Fisher probable least significant difference (PLSD) test and the Student-Newman-Keuls posteriori test were performed. A P value of <0.05 was considered significant.

RESULTS

Effects of Estradiol on ET-1 Synthesis

Detectable amounts of ET-1 were found in the supernatant of the primary cultures of mixed bovine oviduct cells (epithelial cells and fibroblasts) collected after 4 days of culture. ET-1 concentrations were 95–150 pg/mg protein. In the cell cultures treated with 4, 40, 200, and 400 nM of 17β-estradiol, the ET-1 levels decreased in a concentration-dependent manner from 142 ± 14 pg/mg protein (untreated control) to 108.4 ± 11.6, 99 ± 10.3, 80.25 ± 11, and 48.3 ± 8 pg/mg protein, respectively (Fig. 2, bottom). The decreases in ET-1 levels observed were not due to the effects of estradiol on cell growth because the total cellular protein content did not change (Fig. 2, top).

ICI 182,780 Modulates the Inhibitory Effects of 17β-Estradiol on ET-1 Synthesis

To study the modulatory effects of ICI 182,780, cells were pretreated with ICI 182,780 (1 μM). The inhibitory effects of 17β-estradiol (50 ng/ml, 0.2 μM) on ET-1 synthesis were completely reversed in the presence of ICI 182,780 (Fig. 3). Compared with the untreated controls, the ET-1 levels in cell cultures treated with 17β-estradiol decreased from 130.6 ± 15 pg/mg protein to 84.14 ± 12.1 pg/mg protein, whereas in those cultures treated with 17β-estradiol plus ICI 182,780 the ET-1 levels increased to 195 ± 37 pg/mg protein ($P < 0.05$ vs. control; Fig. 3). The inhibitory effects of near physiological concentrations of estradiol (4 nM) on ET-1 synthesis were also completely blocked by 1 μM ICI 182,780. The levels of ET-1 in untreated cells and cells treated with 4 nM estradiol and estradiol plus 1 μM ICI 182,780 were 130.6 ± 14.5, 101 ± 8 ($P < 0.05$ vs. control), and 129 ± 6 pg/mg protein, respectively ($P < 0.05$ vs. estradiol). The changes in ET-1 levels were not due to the effects of the modulatory agents on cell growth because the total cellular protein content did not change (Fig. 3, top).

Effects of Phytoestrogens and PCBs on ET-1 Synthesis

In contrast to the effects of 17β-estradiol, ET-1 synthesis by oviduct cells was significantly induced in the presence of some but not all of the phytoestrogens. Significant increases in ET-1 levels were observed in cells treated with the phytoestrogens biochanin A and genistein. In cultures treated with 2 μM biochanin A and genistein, ET-1 syn-

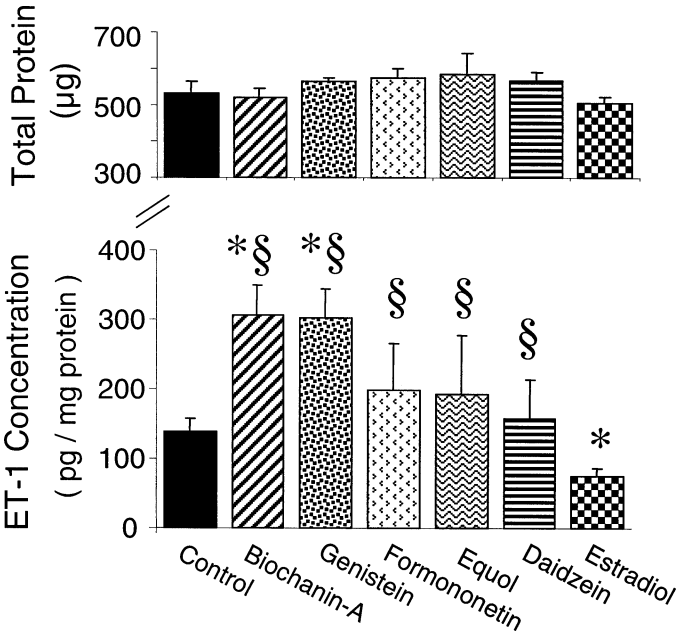

FIG. 4. Effects of 17β-estradiol and phytoestrogens on ET-1 synthesis in cultured bovine oviduct cells (bottom). Confluent monolayers were treated for 4 days with or without 2 μM biochanin A, genistein, formononetin, equol, or daidzein and 0.2 μM 17β-estradiol, and the levels of ET-1 in the medium were assayed. Data represent mean ± SD from four different experiments, each in triplicate, and the amount of ET-1 produced was normalized to total protein concentration. The total protein content (micrograms) in controls and cells treated with biochanin A, genistein, formononetin, equol, daidzein, and 17β-estradiol did not differ and were 531 ± 34, 520 ± 6, 564 ± 10, 575 ± 25, 597 ± 57, 567 ± 23, and 504 ± 18 μg, respectively (top). Symbols indicate significant differences ($P < 0.05$) using Student-Newman-Keuls posteriori test: *$P < 0.05$ vs. control; §$P < 0.05$ vs. estradiol. Similar significance values were obtained by using the Fisher PLSD test. For the ANOVA, F = 14.363 with 6 (treatments) and 24 (residual) degrees of freedom, $P < 0.0001$; lambda = 86.179.

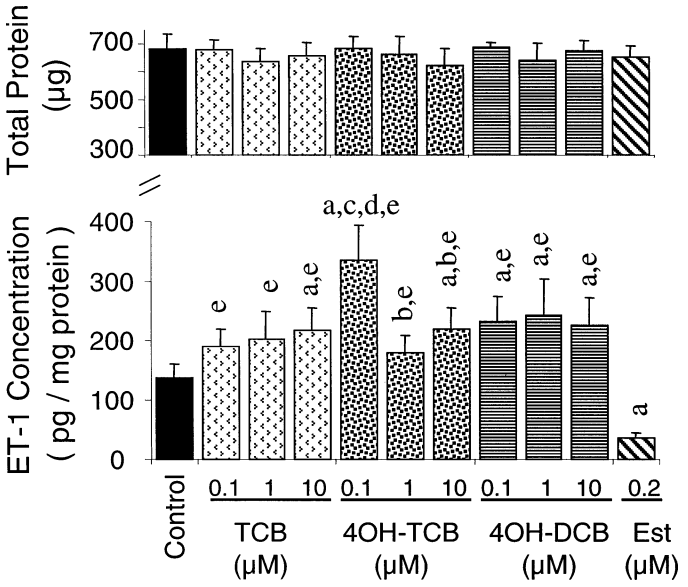

FIG. 5. Effects of various concentrations (0.1–10 μM) of the PCBs (TCB, 4-OH-TCB, and 4-OH-DCB) and 0.2 μM estradiol on the production of ET-1 by confluent monolayers of bovine oviduct cells treated for 4 days (bottom). Data represent mean ± SD from three individual experiments, each in triplicate. The amount of ET-1 produced was normalized to total protein concentration. The total protein content (micrograms) in controls and cultures treated with 0.1, 1, and 10 μM of TCB, 4-OH-TCB, and 4-OH-DCB did not differ and was 681 ± 55, 678 ± 36, 637 ± 47, 658 ± 48, 684 ± 42, 663 ± 63, 621 ± 62, 688 ± 18, 642 ± 61, and 675 ± 38 μg, respectively (top). Letters indicate significant differences ($P < 0.05$) using Student-Newman-Keuls posteriori test: a, $P < 0.05$ vs. control; b, $P < 0.05$ vs. 0.1 μM of TCB or 4-OH-TCB or 4-OH-DCB; c, $P < 0.05$ vs. 1 μM of TCB or 4-OH-TCB or 4-OH-DCB; d, $P < 0.05$ vs. 10 μM of TCB or 4-OH-TCB or 4-OH-DCB; e, $P < 0.05$ vs. estradiol. Similar significance values were obtained by using the Fisher PLSD test. For the ANOVA, F = 9.558 with 10 (treatments) and 22 (residual) degrees of freedom, $P < 0.0001$; lambda = 95.583.

thesis was significantly increased from 139 ± 19 pg/mg protein to 305 ± 45 pg/mg protein ($P < 0.05$ vs. control) and 301.7 ± 43 pg/mg protein ($P < 0.05$ vs. control), respectively (Fig. 4, bottom). In cells treated with 2 μM formononetin, equol, and daidzein, ET-1 levels were not significantly increased, from 139 ± 19 pg/mg protein to 198 ± 68, 192 ± 85.2, and 15 8 ± 55 pg/mg protein ($P > 0.05$ vs. control), respectively. The changes in ET-1 levels were not due to the effects of the modulatory agents on cell growth because the total cellular protein content did not change (Fig. 4, top).

Similarly, ET-1 synthesis was also significantly stimulated in bovine oviduct cells treated with the xenoestrogens TCB (0.1–10 μM), 4-OH-TCB (0.1–10 μM), and 4-OH-DCB (0.1–10 μM). The lowest concentrations of TCB, 4-OH-DCB, and 4-OH-TCB that significantly induced ET-1 synthesis were 10 μM, 0.1 μM, and 0.1μM, respectively. Stimulatory effects of TCB on ET-1 synthesis were observed at 10 μM but not at lower concentrations of 0.1 and 1 μM (Fig. 5, bottom). Compared with the untreated controls, ET-1 increased from 137 ± 23 pg/mg protein to 218 ± 38 pg/mg protein in cells treated with 10 μM TCB ($P < 0.05$), 334 ± 60 pg/mg protein in cells treated with 0.1 μM 4-OH-TCB ($P < 0.05$), and 233 ± 41 pg/mg protein in cells treated with 0.1 μM 4-OH-DCB ($P < 0.05$; Fig. 5, bottom). The stimulatory effects of 4-OH-TCB and 4-OH-DCB were more potent than that of TCB. The changes in ET-1 levels were not due to the effects of the modulatory agents on cell growth because the total cellular protein content did not change (Fig. 5, top).

ICI 182,780 Modulates Phytoestrogen- and PCB-Induced ET-1 Synthesis

The stimulatory effects of biochanin A (0.2 μM) and genistein (2 μM) on ET-1 synthesis were differentially modulated by ICI 182,780 (1 μM). In the presence of ICI 182,780 the stimulatory effects of biochanin A on ET-1 synthesis were decreased from 436 ± 78 to 334 ± 108 pg/mg protein and the stimulatory effects of genistein on ET-1 synthesis were increased from 299 ± 30.3 to 351 ± 47 pg/mg protein (Fig. 6, bottom). The changes in ET-1 levels were not due to the effects of the modulatory agents on cell growth because the total cellular protein content did not change (Fig. 6, top).

In the presence of ICI 182,780, the stimulatory effect of 0.1 μM 4-OH-TCB was reduced from 334 ± 74 to 203 ± 47 pg/mg protein ($P < 0.05$) and the effects of 0.1 μM 4-OH-DCB were increased from 233 ± 53 to 330 ± 17.4 pg/mg protein ($P < 0.05$). ICI 182,780 also increased the stimulatory effects of TCB (0.1 μM) on ET-1 synthesis from 190.5 ± 28.5 to 274 ± 71 pg/mg protein ($P < 0.05$; Fig. 7, bottom). The changes in ET-1 levels were not due to the effects of the modulatory agents on cell growth because the total cellular protein content did not change (Fig. 7, top).

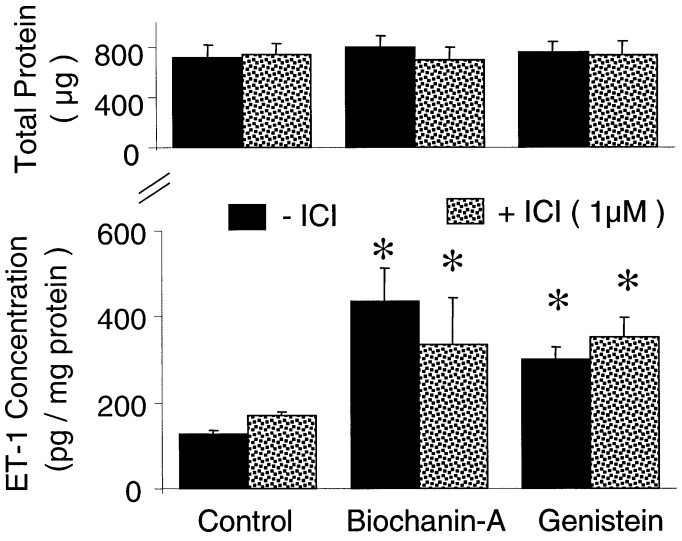

FIG. 6. Modulatory effects of ICI 182,780, an ER antagonist, on biochanin A (Bioch-A)- and genistein-induced ET-1 synthesis by confluent monolayers of bovine oviduct cells. Cells were treated for 4 days with or without biochanin A 0.2 μM or genistein 2.0 μM with or without 1 μM ICI 182,780, and ET-1 levels were assayed in the culture medium (bottom). Data represent mean ± SEM from three individual experiments, each conducted in triplicate. The amount of ET-1 was normalized to total protein concentration. The total protein content (micrograms) in controls and cells treated with ICI, biochanin-A, biochanin-A + ICI, genistein, and genistein + ICI, did not differ and was 716 ± 100, 740 ± 85, 796 ± 92, 696 ± 104, 762 ± 77, and 734 ± 113 μg, respectively (top). For the Student-Newman-Keuls posteriori test, $*P < 0.05$ vs. control. Similar significance values were obtained by using the Fisher PLSD test. For the ANOVA, F = 11.516 with 5 (treatments) and 12 (residual) degrees of freedom, $P < 0.0003$; lambda = 57.58.

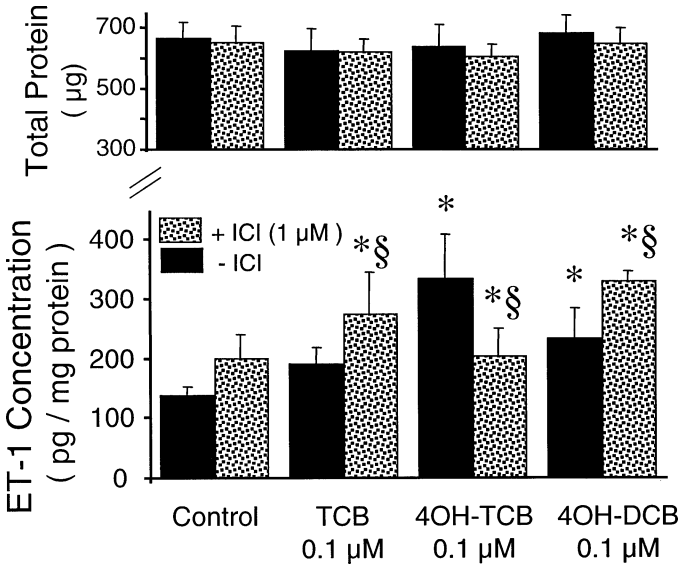

FIG. 7. Modulatory effects of 1μ M ICI 182,780, a selective ER antagonist, on (0.1 μM) TCB-, 4-OH-TCB-, and 4-OH-DCB-induced ET-1 production by confluent monolayers of oviduct cells treated for 4 days (bottom). Data represent mean ± SD from three separate experiments, each in triplicate, and the amount of ET-1 was normalized to total protein concentration. The total protein content (micrograms) in controls and cells treated with ICI, TCB, TCB + ICI, 4-OH-TCB, 4-OH-TCB + ICI, 4-OH-DCB, and 4-OH-DCB + ICI did not differ and was 664 ± 54, 651 ± 54, 624 ± 73, 619 ± 43, 637 ± 73, 605 ± 40, 682 ± 57, and 647 ± 52 μg, respectively (top). Symbols indicate significant differences ($P < 0.05$) using Student-Newman-Keuls posteriori test: $*P < 0.05$ vs. control; §$P <$ 0.05 vs. treatment without ICI. Similar significance values were obtained by using the Fisher PLSD test. For the ANOVA, F = 5.468 with 7 (treatments) and 15 (residual) degrees of freedom, $P < 0.0028$; lambda = 38.27. $*P < 0.05$ vs. control; $P < 0.05$ vs. cells treated with the compounds in combination with ICI.

ERα and ERβ Expression and Binding Studies

Western blot analysis of oviduct cell lysates showed that both ERα and ERβ are highly expressed in the oviduct cells (Fig. 8). Binding studies revealed that [^{3}H]17β-estradiol binds with high affinity and specificity to ERs in oviduct cells. The binding isotherm showed a saturable binding process, and the Scatchard analysis revealed that the number of binding sites ranged between 16.5 and 19 fmol/mg protein (Fig. 8, B and C) with a dissociation constant (K_d) of 3.58 ± 0.7 nM.

DISCUSSION

The results of the present study demonstrate that under basal conditions mixed cultures of bovine oviduct epithelial cells and fibroblasts synthesized ET-1. Treatment with estradiol inhibits the basal synthesis of ET-1 in a concentration-dependent manner. Moreover, the inhibitory effects of estradiol on ET-1 synthesis were reversed in the presence of ICI 182,780, a specific ER antagonist [11]. The inhibitory effects of estradiol on ET-1 synthesis were not mimicked by xenoestrogens and the phytoestrogens biochanin-A and genistein. In contrast to the effects of 17β-estradiol, ET-1 synthesis by oviduct cells was dramatically increased by both phytoestrogens and xenoestrogens. Moreover, the stimulatory effects of both phytoestrogens and xenoestrogens were not blocked in the presence of the ER blocker ICI 182,780. These findings provide evidence that estradiol inhibits ET-1 synthesis in oviduct cells and that these effects are ER mediated. In contrast to estradiol, the stimulatory effects of EEs on ET-1 synthesis are mediated via an ER-independent mechanism. Considering the fact that ET-1 regulates oviduct contractility [8] and is a potent mitogen, ET-1 may play a role in regulating oviduct biology/physiology. The finding that estradiol inhibits whereas EEs induce ET-1 synthesis suggests that the contrasting effects of EEs on ET-1 synthesis may be of pathophysiological importance. EEs may induce deleterious effects on the repro-

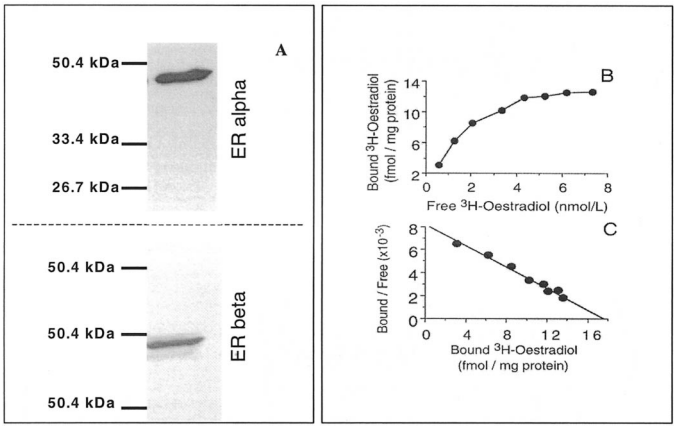

FIG. 8. **A**) Western blots of protein extracts (25 μg/lane) from cultured bovine oviduct cells depicting the presence of ERα (top) and ERβ (bottom). **B**) Binding isotherm for ERs in cultured bovine oviduct cells. Data are from a representative culture and show specifically bound [^{3}H]17β-estradiol (fmol/mg protein) vs. free [^{3}H]17β-estradiol (nmol/L). **C**) Scatchard analysis of specific [^{3}H]17β-estradiol binding in oviduct cells from a representative experiment.

ductive system by influencing physiological processes regulated by ET-1 in response to estradiol.

In the oviduct, the synchronized processes of fertilization, embryo transport, and implantation are controlled by the cyclic release of sex hormones such as estradiol [6]. Therefore, the influence of estradiol on ET-1 synthesis may be of physiological and biological relevance. In this context, the local release of ET in the oviduct may be involved in the cyclic contractions regulated by ovarian hormones. Treatment of uterine segments in vitro with hormones reduces ET-induced contractions [12]. In bovine endothelial cells, ET-1 synthesis is attenuated by estradiol [13, 14].

In the present study, a mixed culture system of oviduct epithelial and fibroblast cells was used to evaluate the effects of estradiol and EEs to reflect in vivo physiology. In the female reproductive tract, ERs are located in the stroma cells [15, 16], and interactions between epithelial cells and fibroblasts play an important role in maintaining oviductal biology and physiology. Factors synthesized by fibroblasts influence epithelial cell function in an autocrine/paracrine fashion in the female reproductive tract [17].

Basal synthesis of ET-1 by oviduct cells was inhibited by estradiol. Similar inhibitory effects of estradiol on ET-1 synthesis have been observed in vascular endothelial cells [14]. Our observations that the oviduct cells express both ERα and ERβ and that the inhibitory effects of estradiol were completely reversed in the presence of the ER antagonist ICI 182,780 (which binds with equal affinity to ERα and ERβ) provide direct evidence that the inhibitory effects were ER mediated. However, the individual roles of ERα and ERβ in mediating the inhibitory effects of estradiol are still unclear. In contrast to estradiol, genistein, a phytoestrogen with high affinity for ERβ (98% binding affinity for ERβ and 4% binding affinity for ERα [11]), did not inhibit ET-1 synthesis. This finding suggests that the inhibitory effects of estradiol on ET-1 synthesis are potentially ERα mediated. This notion is further supported by the fact that the modulatory effects of estradiol, but not genistein, were blocked by ICI 182,780.

In the presence of ICI 182,780, the inhibitory effects of estradiol on ET-1 synthesis were not blocked but were stimulated. This finding implies that the oviduct cells may synthesize endogenous estrogens or estradiollike molecules that inhibit ET-1 synthesis via ERs. Basal synthesis of ET-1 was consistently increased by 20–30% in oviduct cells treated with ICI 182,780, although this increase did not reach statistical significance. Aromatase, a key enzyme in the synthesis of estradiol, is present in multiple tissues, including the oviduct [18]. Thus, local synthesis of estradiol or estradiollike molecules is plausible and may negatively regulate ET-1 synthesis via ER-dependent mechanisms. Alternatively, factors in 1% FCS (charcol stripped, hormone free) used in this study may have stimulated ET-1 synthesis via ERs, and this effect would be blocked by ICI 182,780. Growth factors induce the estrogen response element [19], which mediates most of the ER-mediated effects.

In contrast to estradiol, phytoestrogens induced ET-1 synthesis in oviduct cells, and these effects were not positively correlated with their binding affinity to ERs, suggesting that these stimulatory effects were mediated via ER-independent mechanisms. Biochanin A, a precursor of genistein with 10 000 fold less binding affinity for both ERα and ERβ [11], was more potent than genistein, a ERβ ligand, in inducing ET-1 synthesis. This finding further supports the hypothesis that phytoestrogens mediate their stimulatory effects on ET-1 synthesis via some ER-independent mechanism. Similar to phytoestrogens and in contrast to estradiol, the PCBs TCB, 4-OH-TCB, and 4-OH-DCB induced ET-1 synthesis in oviduct cells. Again, the potency of the PCBs in inducing ET-1 synthesis was not correlated with their binding affinity to ERs. The stimulatory effects of PCBs were not blocked by ER antagonist ICI 182,780, suggesting that the stimulatory effects were mediated via an ER-independent mechanism(s). In this context, xenoestrogens and phytoestrogens bind to arylhydrocarbon (Ah) receptors, and these receptors play a critical role mediating the antiestrogenic effects of EEs by modulating their metabolism [9, 19]. Therefore, we hypothesize that the stimulatory effects of EEs on ET-1 synthesis are Ah receptor mediated; however, this possibility needs to be explored.

Apart from the Ah receptor, other mechanisms may also be involved in mediating the stimulatory effects of EEs on ET-1 synthesis. PCBs stimulate the release of free radicals [9], which are known to stimulate ET-1 synthesis. PCBs also inhibit the free radical scavenging systems, i.e., superoxide dismutase and reduced glutathione [9], which may further potentiate the effects of free radicals on ET-1 synthesis. Phytoestrogens are known to bind to a newly identified functional type II ER [9], which regulates cell growth and function, and may be responsible for mediating its stimulatory effects on ET-1 synthesis. Studies are needed to elucidate the exact mechanism(s) involved in these actions.

The lowest concentrations of PCBs and phytoestrogens that induced ET-1 synthesis were 0.1 μM and 0.2 μM, respectively. At a similar concentration, i.e., 0.2 μM, the inhibitory effects of estradiol on ET-1 synthesis were much lower than the stimulatory effects of EEs on ET-1 synthesis. PCBs accumulate in the body to a greater extent and so may have a stronger impact on the reproductive system. PCBs are relatively new, synthetic estrogens, whereas consumption of phytoestrogens has coevolved with hormonal regulation of the reproductive system. Under in vivo situations, the total plasma concentrations of phytoestrogens (equol, daidzein, formononetin, genistein, biochanin A) in humans consuming a soya-rich diet is 1.8 μM [9, 20]. However, on a normal/low-soya diet phytoestrogen concentrations may be relatively low. Because high concentrations of phytoestrogens and EEs are associated with reproductive disorders, continuous exposure to EEs and the presence of EEs within the body may result in increased ET-1 synthesis and may interfere with the cyclic, time-dependent physiological regulation of the oviduct, a key organ for reproductive processes. Because estradiol and phytoestrogens bind to ERs but have contrasting effects on ET-1 synthesis, presence of these EEs may block the effects of estradiol by competing for the ER.

In a previous study, we provided the first evidence that phytoestrogens and xenoestrogens mimic the effects of estradiol in inducing the synthesis of leukemia inhibitory factor by oviduct cells. We also provided evidence that these effects were mediated via an ER-dependent mechanism(s) [20] and potentially via ERβ because genistein, an ERβ ligand, induced leukemia inhibitory factor synthesis. In contrast to our previous findings, in the present study phytoestrogens and xenoestrogens did not mimic the effects of estradiol on ET-1 synthesis but rather had contrasting effects. Taken together these results suggest that EEs can modulate the synthesis of autocrine/paracrine factors via multiple mechanisms and via ER-dependent and ER-independent mechanisms. These effects could be estrogenic or antiestrogenic. Future studies are required to elucidate the

various mechanisms by which EEs may directly or indirectly influence the reproductive system.

These findings provide the first evidence that ET-1 synthesis in oviduct cells is regulated by the ovarian hormone estradiol. The inhibitory effect of estradiol on ET-1 synthesis is ER mediated. EEs (phytoestrogens and xenoestrogens) do not mimic the inhibitory effects of estradiol on ET-1 synthesis but rather stimulate ET-1 synthesis, and these stimulatory effects on ET-1 synthesis are potentially mediated via an ER-independent mechanism. These contrasting effects of EEs on ET-1 synthesis may interfere with estradiol-mediated processes and may act as endocrine disrupters, possibly inducing deleterious effects on the reproductive system by influencing the biology and physiology of the oviduct and potentially leading to infertility.

REFERENCES

1. Sayegh R, Mastroianni L. Recent advances in our understanding of tubal function. Ann N Y Acad Sci 1991; 626:266–275.
2. Rosselli M, Dubey RK, Rosselli MA, Macas E, Fink D, Lauper U, Keller PJ, Imthurn B. Identification of nitric oxide synthase in human and bovine oviduct. Mol Hum Reprod 1996; 2:607–612.
3. Nebekura H, Koike H, Ohtsuka T, Yamaguchi M, Miyakawa I, Mori N. Fallopian tube prostaglandin production with and without endometriosis. Int J Fertil Menopausal Stud 1994; 39:57–63.
4. Rosselli M, Imthurn B, Macas E, Keller PJ. Endothelin production by bovine oviduct epithelial cells. J Reprod Fertil 1994; 101:27–30.
5. Dubey RK, Rosselli M, Imthurn B, Keller PJ, Jackson EK. Vascular effects of environmental estrogens: implications for reproductive and vascular health. Hum Reprod Update 2000; 6:351–363.
6. Harper MJK. Gamete and zygote transport. In: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 103–134.
7. Reinhart KC, Dubey RK, Mummery CL, van Rooijen M, Keller PJ, Rosselli M. Synthesis and regulation of leukemia inhibitory factor in cultured bovine oviduct cells by hormones. Mol Hum Reprod 1998; 4:301–308.
8. Rosselli M, Imthurn B, Macas E, Keller PJ, Dubey RK. Endogenous nitric oxide modulates endothelin-1 induced contractions of bovine oviduct. Biochem Biophys Res Commun 1994; 20:143–148.
9. Rosselli M, Reinhart K, Imthurn B, Keller PJ, Dubey RK. Cellular and biochemical mechanisms by which environmental estrogens influence reproductive function. Hum Reprod Update 2000; 6:332–350.
10. Reinhart KC, Dubey RK, Keller PJ, Lauper U, Rosselli M. Xenoestrogens and phytoestrogens induce the synthesis of inhibitory factor by human and bovine oviduct cells. Mol Hum Reprod 1999; 5:899–907.
11. Kuiper GG, Carlsson B, Gandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor-alpha and beta. Endocrinology 1997; 138:863–870.
12. McGovern PG, Goldsmith LT, Schmidt CL, Von Hagen S, Linden M, Weiss G. Effect of endothelin and relaxin on rat uterine segment contractility. Biol Reprod 1992; 46:680–685.
13. Akishita N, Kozaki K, Eto M, Yoshizumi M, Ishikawa M, Toba K, Orimo H, Ouchi Y. Estrogen attenuates endothelin-1 production by bovine endothelial cells via estrogen receptor. Biochem Biophys Res Commun 1998; 251:17–21.
14. Dubey RK, Jackson EK, Keller PJ, Imthurn B, Rosselli M. Estradiol metabolites inhibit endothelin synthesis by an estrogen receptor-independent mechanism. Hypertension 2001; 37(part 2):640–644.
15. Cooke PS, Buchanan DL, Lubahn DB, Cunha GR. Mechanisms of estrogen action: lessons from the estrogen receptor-alpha knockout mouse. Biol Reprod 1998; 59:470–475.
16. Osteen KG, Rogers WH, Gaire M, Hargrove JT, Gorstein F, Matrisian LM. Stromal-epithelial interactions mediate steroidal regulation of metalloproteinase expression in human endometrium. Proc Natl Acad Sci U S A 1994; 91:10129–10133.
17. Taylor AH, Al-Azzawi F. Immunolocalization of estrogen receptor beta in human tissues. J Mol Endocrinol 2000; 24:145–155.
18. Martel C, Melner MH, Gagne D, Simard J, Labrie F. Widespread tissue distribution of steroid sulfatase, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD), 17 beta-HSD 5 alpha-reductase and aromatase activities in the rhesus monkey. Mol Cell Endocrinol 1994; 104:103–111.
19. Safe SH. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. CRC Crit Rev Toxicol 1994; 24:87–95.
20. Dubey RK, Gillespie DG, Imthurn B, Rosselli M, Jackson EK, Keller PJ. Phytoestrogens inhibit growth and MAP kinase activity in human aortic smooth muscle cells. Hypertension 1999; 33:177–182.