Differing Effects of Endocrine-Disrupting Chemicals on Basal and FSH-Stimulated Progesterone Production in Rat Granulosa-Luteal Cells

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Previous studies have shown that the phytoestrogen, genistein, inhibits basal and forskolin-stimulated progesterone synthesis in rat granulosa-luteal cells. Genistein, however, not only binds and activates the estrogen receptor (ER), but is also a potent inhibitor of tyrosine kinase. In these studies we have compared the effects of estradiol, two other phytoestrogens, apigenin and coumarin, the pesticide, [2-(chlorphenyl)-2-(4-chlorphenyl)-1,1,1-trichlorethan] (2,4'DDT), and the industrial chemical, 4-octyl-phenol, on basal and follicle stimulating hormone (FSH)-stimulated progesterone production in the same experimental system. Only a supraphysiological dose of estradiol (10^{-5} M) significantly inhibited basal and forskolin-stimulated progesterone production in granulosa-luteal cells, but had no effect on FSH-stimulated production. In contrast, apigenin, DDT, and octyl-phenol stimulated basal progesterone production at doses around 10^{-8} to 10^{-7} M, but this effect was reversed at higher doses. Coumarin was without effect. Like basal production, the two phytoestrogens had opposing effects on FSH-stimulated progesterone production. Genistein at 10^{-5} M was inhibitory, while apigenin significantly potentiated the response at 19^{-7} M. In contrast, DDT had no effect on the FSH-induced response, though 10^{-7} M octyl-phenol nearly doubled the response. While all these chemicals are known to interact with the estrogen receptor to a greater or lesser extent, these studies suggest that like genistein, these different endocrine-disrupting chemicals may have other actions apart from those on the estrogen receptor.

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There is increasing interest and debate concerning the possible health risks and benefits of environmental estrogens, particularly with regard to the organochlorine pesticides, industrial chemicals, and the phytoestrogens (1–3). Numerous studies have shown that such chemicals do interact with estrogen receptors (ER's), albeit at concentrations at least 100- to 1000-fold lower than estradiol, and can stimulate transcriptional activity in transfected cells (4–6). Their *in vivo* effects, however, at different potential target tissues remains an issue of much speculation. *In utero* and neonatal exposure of animals to environmental estrogens can cause abnormalities in reproductive processes (7–9) and there is circumstantial evidence that phytoestrogens may have beneficial effects in women (10, 11). Whether these effects are due to environmental estrogens acting as estrogen agonists or antagonists of endogenous estrogens is not known. Alternatively, they may be acting via a nonestrogenic pathway.

In 1987, Akiyami *et al.* (12) demonstrated that genistein, one of the most potent phytoestrogens and one that is present in high concentrations in soy, inhibited protein tyrosine kinase activity and the action of epidermal growth factor. Recent experiments in our laboratory demonstrated that genistein and another nonestrogenic tyrosine kinase inhibitor, lavendustin A, dose-dependently inhibited progesterone production in cultured rat granulosa cells (13). The drugs did not reverse the cytokine-induced inhibition of progesterone synthesis. Inhibition of human chorionic gonadotrophin (hCG)-induced progesterone production in Leydig tumor cells by the industrial pollutants, bisphenol A and octyl-phenol, as well as estradiol and diethylstilboestrol has also been reported recently (14).

Estrogen receptors (ERs), notably ERβ, are present on granulosa cells (15, 16) and estrogens are known to regulate granulosa cell function (17). We have thus investigated the effects of different environmental estrogens and the native 17β estradiol on progesterone synthesis in primary cultures of rat granulosa cells. The known effects of genistein, an

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isolfavone, have been compared with two other phytoestrogens that have different estrogenic activities. The flavone derivative, apigenin, has lower binding and transcriptional activity at the ER (6) than that of genistein (up to 10- and 4-fold lower, respectively), while coumeran, unlike coumestrol (6), has virtually none, if any, estrogenic activity and was used as a form of negative control. The two pollutants, the pesticide, [2-(chlorphenyl)-2-(4-chlorphenyl)-1,1,1-trichlorethan] 2,4'DDT, and the industrial chemical, 4-octyl-phenol, were investigated because of the evidence of their effects on sexual differentiation, development, and function in wildlife populations and laboratory animals (2).

Materials and Methods

Animals. Adult female Porton Wistar rats (200–250 g) were housed under controlled conditions of light (lights from 06:00–18:00 hr) and temperature (20°C) and had free access to food and water. Daily vaginal smears determined normal 4-day estrous cycles and only those with at least two consecutive regular cycles were used for the experiments. On the morning of estrus (08.30–09.00 hr), three to four rats were sacrificed by stunning and decapitation (approved by St. George's Hospital Medical School Ethical Review Committee) and their ovaries were dissected and placed in ice-cold saline.

Cell Cultures. Freshly ruptured follicles were dissected microscopically in ice-cold saline and placed in culture medium containing 2 mg/ml collagenase, 10 μg/ml deoxyribonuclease, and 10 mg/ml bovine serum albumin (BSA; all from Sigma, Poole, Dorset, UK). After 10 to 15 min and passage through a 25-gauge needle, cells were washed twice and plated in 250-μl aliquots in 96-well culture plates at a concentration of 3×10^5 cells/ml. The culture medium was McCoys 5A containing 25 mM HEPES, 2 mM glutamate, 0.1% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma). Cells were cultured for 24 hr with 5% fetal calf serum (Sigma) and for an additional 48 hr with serum-free medium and in the presence of appropriate drugs. Media samples were taken and stored at −20°C, and cellular viability was assessed by the trypan blue exclusion test of by assessment of cellular dehydrogenases using the ability of cells to convert 3-[4,5-dimethylthrazol-2-yl]-2,5-diphenyltetrasolium bromide (MTT) into formazan during a 4-hr incubation (18).

Drugs. The following drugs were used in these experiments: genistein (4', 5,7-trihydroxyisoflavone), apigenin (4', 5,7-trihydroxyflavone), coumarin (1,2-benzopyrone), 2,4'DDT, 4-octyl-phenol, 17β-estradiol, forskolin, and ovine FSH. With the exception of FSH, kindly supplied by The National Hormone and Pituitary Agency (Torrance, CA), all other drugs were supplied by Sigma. Steroids and estrogenic drugs were initially dissolved in alcohol and diluted appropriately with culture medium before they were stored in aliquots as stock solutions. Aliquots were diluted appropriately for each experiment and drugs were added to cultures in 10-μl volumes to give the desired final concentration. Controls were performed to ensure that the maximum volume of ethanol diluent did not affect cellular responses.

Steroid Assays. Progesterone concentrations in the medium were measured in duplicate by a direct radioimunnoassay (RIA) kit (ICN Pharmaceuticals, Basingstoke, Buckinghamshire, UK) according to the manufacturer's instructions. All drugs used in these experiments were tested for their possible cross reactivity with the antisera used, but none was detected. The cross reactivity of the progesterone antiserum with 20α-dihydroprogesterone, 17α-hydroxyprogesterone, and pregnenolone was 5.4%, 0.6%, and 0.4%, respectively, and with estradiol β was <0.01. There was no cross reactivity of any of the drug with the progesterone antiserum. Inter- and intraassay coefficients of variation were 5.9% and 3.0%, respectively.

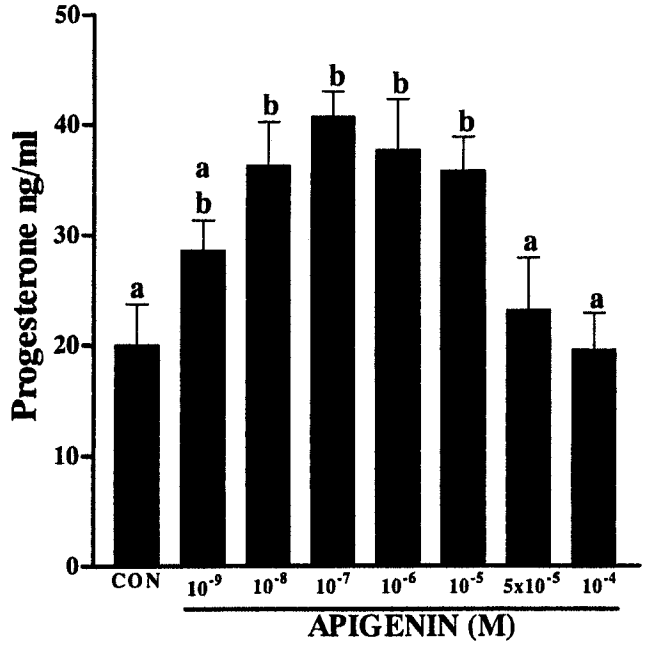
Statistical Analysis. Data shown represent means ± SEM of triplicate cultures obtained from at least three independent experiments and n is the total number of individual observations. Control values are those that have been paired with the appropriate drug responses. Statistical differences in the dose responses were compared with an analysis of variance followed by Gabriel's test that is suitable for groups of unequal size. A paired Student's t test was used when only two groups were compared.

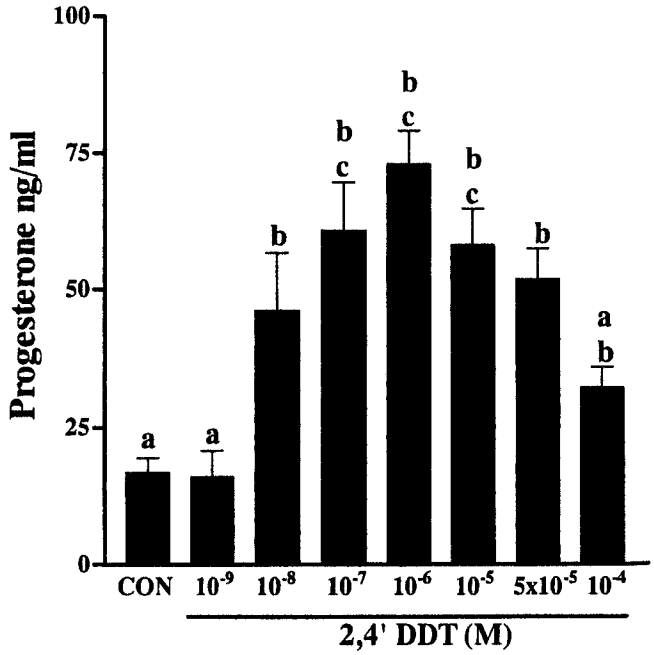
Results

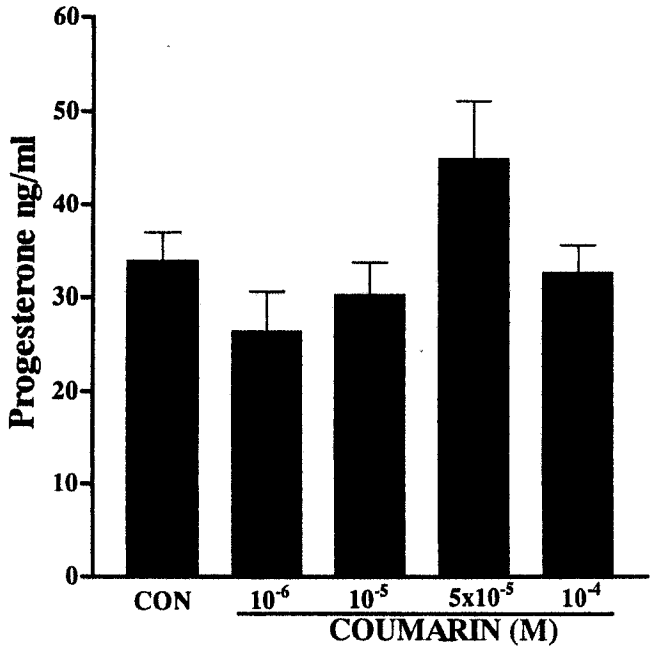
Our previous studies (13) reported that the phytoestrogen, genistein, induced a significant inhibition of basal progesterone production at a dose of 5×10^{-7} M. This contrasts the effects of two other phytoestrogens investigated in the present experiments. Apigenin induced a biphasic dose response with significant stimulation of basal progesterone production observed at doses between 10^{-8} and 10^{-5} M, but at 5×10^{-5} and 10^{-4} M, progesterone production was similar to control values (Fig. 1). Coumarin had no effect on basal progesterone secretion at any of the doses tested (10^{-6} to 10^{-4} M) (Fig. 1.)

Like apigenin, both 2,4'DDT and 4-octyl-phenol significantly stimulated basal progesterone production with maximal stimulatory effects being observed at 10^{-6} and 10^{-5} M, respectively (Fig. 2). At 10^{-4} M there was a reduction of the stimulatory effect of both drugs and though the mean concentrations were higher than the control values, they were not statistically different. Estradiol significantly ($P < 0.05$) inhibited basal progesterone production, but only at the highest concentration of 10^{-5} M, which is well beyond the physiological concentration of this hormone (Fig. 3). Estradiol also inhibited the response to forskolin, but, again, only significantly at the highest dose tested (Fig. 3). It had no effect on FSH-stimulated progesterone production (results not shown).

The effects of apigenin, genistein, 2,4'DDT, and 4-octyl-phenol on FSH-stimulated progesterone secretion are shown in Figures 4 and 5. Apigenin potentiated the proges-







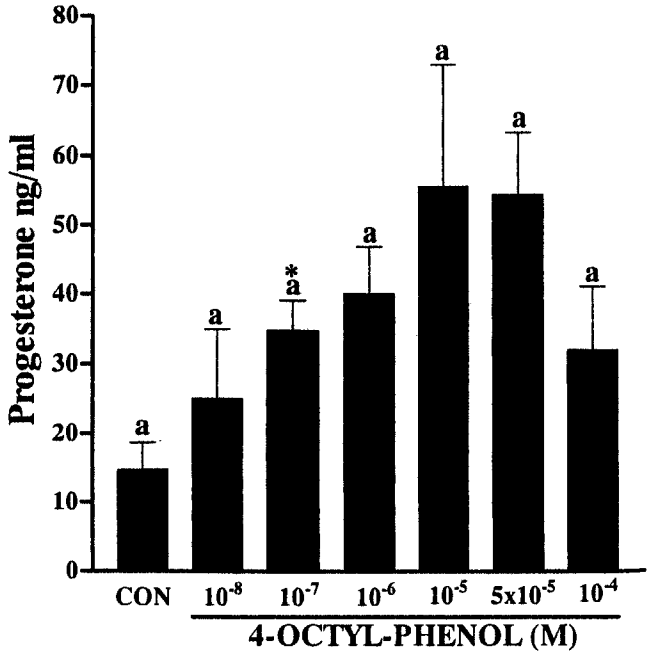


Figure 1. Dose response profiles of basal progesterone production in granulosa-luteal cells cultured for 48 hr in the presence or absence (Con) of different molar doses of apigenin and coumarin as indicated on the histograms. Data shows the means ± SEM of nine to 12 observations and different letters denote statistical significance ($P < 0.05$, Gabriel's test).

Figure 2. Dose response profiles of basal progesterone production in granulosa-luteal cells cultured for 48 hr in the presence or absence (Con) of different molar doses of DDT and 4-octyl-phenol as indicated on the histograms. Data shows the means ± SEM of nine to 12 observations and different letters denote statistical significance ($P < 0.05$, Gabriel's test). *$P < 0.005$ compared with the control observations (Student's t test).

terone response to FSH at 10^{-5} and 10^{-7} M, the lower dose being more potent in this respect. In contrast, genistein at doses of 10^{-5} and 5×10^{-5} M inhibited FSH-induced progesterone production in a dose-dependent manner, but a dose of 10^{-6} M was ineffective in this respect (results not shown). The effect of coumarin on FSH-induced progesterone production was not investigated.

2,4'DDT and 4-octyl-phenol had differing effects on FSH-stimulated progesterone production. 2,4'DDT at 10^{-6} and 10^{-8} M did not increase the response beyond that ob-

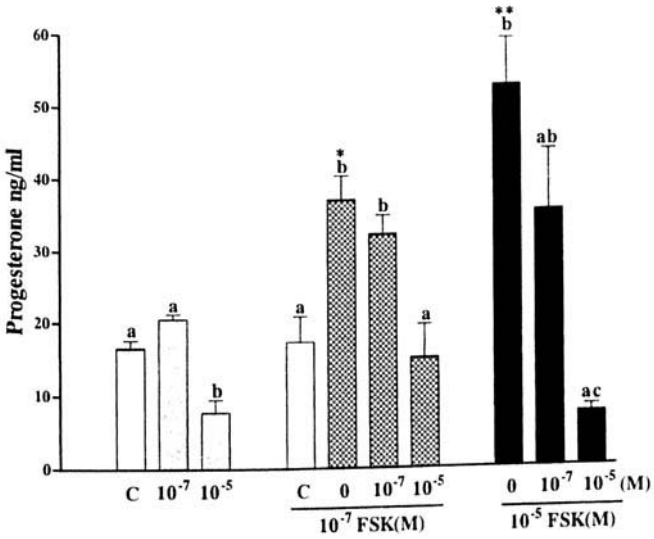


Figure 3. Effects of estradiol on basal and forskolin-stimulated progesterone production in granulosa-luteal cells. Cells were cultured for 48 hr in the presence of 10^{-7} and 10^{-5} M estradiol (first block) or with the same doses of estradiol plus either 10^{-7} or 10^{-5} M forskolin (FSK). C represents the appropriate paired controls (they were the same for both FSK groups) and 0 represents the progesterone response to FSK in the absence of estradiol. Values are means ± SEM of nine to 12 observations. For each group of results different letters denote statistical significance of $P < 0.05$ (Gabriel's test) and * and ** $P < 0.01$ and 0.001, respectively, compared with the corresponding control (C) value (Student's *t* test).

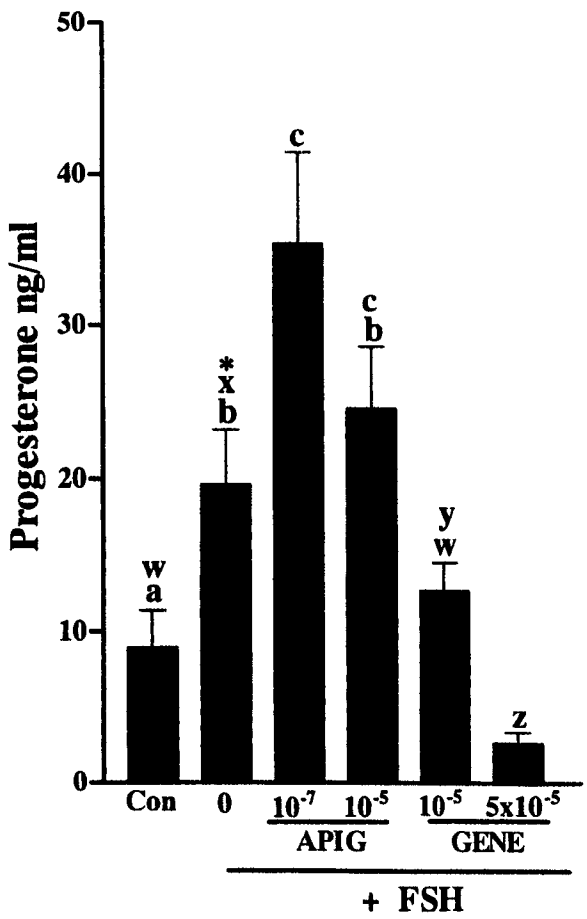


Figure 4. The effects of 10^{-7} and 10^{-5} M apigenin (APIG) and genistein (GENE) on the progesterone response to 10 ng/ml FSH. Granulosa cells were cultured for 48 hr in the absence (Con) of any drug, with FSH alone (0) or FSH plus the phytoestrogen. Data are means ± SEM of eight to nine observations and different letters indicate statistical significance ($P < 0.05$, Gabriel's test. a, b, and c compares the data obtained for apigenin and w, x, y, and z the data obtained with genistein. *$P < 0.01$ compared with the control value (Student's *t* test).

served with FSH alone (Fig. 5). Indeed, it may even have blocked the FSH response in light of the fact that both doses of the drug stimulated basal progesterone secretion with a magnitude similar to that observed with FSH alone. In contrast, 4-octyl-phenol at doses of 10^{-7} and 10^{-5} M markedly potentiated the progesterone response to FSH (Fig. 5).

Table I shows the data obtained from the cell viability assessments that were made at the end of the culture period. Only the highest dose (100 μM) of apigenin and 4-octyl-phenol had any marked effects on cell viability, as assessed by their ability to reduce MTT.

Discussion

Many of the endocrine-disrupting chemicals have been shown to competitively bind to the estrogen receptor and, like estradiol, to initiate gene transcription in transfected cells (6). Their estrogenic activity has also been screened by their ability to increase cell division in an estrogen-receptor-positive breast cancer cell line (5, 19). They have been classified as xenoestrogens.

While such screening procedures provide valuable evidence for the potential potency of such xenoestrogens, their possible toxicological/beneficial effects *in vivo* may not necessarily be mediated through competitive binding with the estrogen receptor. Indeed, the discovery that genistein was a potent tyrosine kinase inhibitor could explain why the incidence of certain cancers is significantly lower among populations that consume high concentrations of soy (20).

Historically speaking, the first evidence for endocrine-disrupting chemicals came from studies that showed a correlation between pollution and abnormal sexual differentiation and development in certain wildlife populations (21). This stimulated controlled laboratory studies investigating the effects of neonatal and/or early postnatal exposure to endocrine-disrupting chemicals on sexual development and reproductive competence. While such experiments generally support the wildlife observations, there is no direct evidence that these effects are mediated by their ability to bind with the estrogen receptor, thereby potentiating or antagonizing endogenous estrogens. Indeed, the estrogenicity of different endocrine-disrupting chemicals certainly differs when tested in different assays or screening systems.

The current studies were initiated by our finding that genistein was a potent inhibitor of steroidogenesis, although it had been used as a tool for inhibiting tyrosine kinase activity, not as a xenoestrogen *per se*. The relative binding affinity of genistein at the estrogen receptor β (ERβ) and ERα compared with estradiol itself (arbitrarily set at 100)

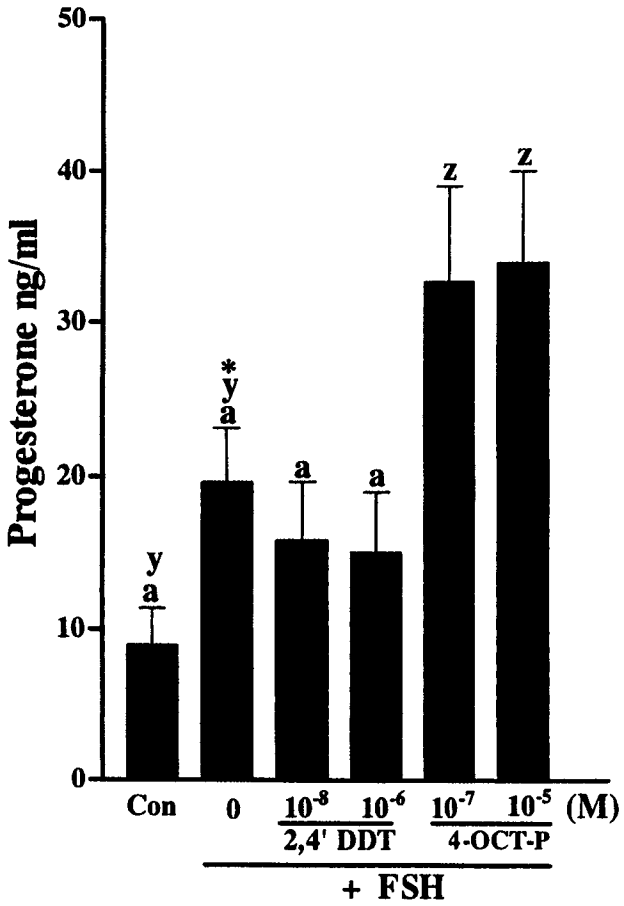


Figure 5. The effects of 10^{-8} and 10^{-6} M DDT and 4-octyl-phenol (OCT-P) on the progesterone response to 10 ng/ml FSH. Granulosa cells were cultured for 48 hr in the absence (Con) of any drug, with FSH alone (0) or FSH plus DDT or OCT-P. Data are means ± SEM of nine observations and different letters indicate statistical significance ($P < 0.05$, Gabriel's test). a and b compares the data obtained for DDT and y and z compares the data obtained with OCT-P. *$P <$ 0.001 compared with the control value (Student's *t* test).

has been reported to be 17% and 0.7% (6), although another study quoted a value of 0.1% with no distinction made between the α and β receptors (5). In terms of its transcriptional activity, genistein is weak compared with estradiol. In cotransfected cultures of human embryonal kidney cells, maximal transcriptional activity was obtained with 10^{-10} M estradiol, while similar activity was only observed with doses of genistein approximately 100-fold higher (6). Other studies designed to test the bioactivity of genistein compared with that or estradiol have also shown that its relative potency is between 100- and 1000-fold lower (4).

It is, however, noteworthy that at a dose of 10^{-7} M, genistein induced greater transcriptional activity than that of estradiol, reaching a maximum at approximately 200% (estradiol arbitrarily set at 100%) at 10^{-6} M (6). Thus, at high concentrations, genistein may have been inducing the same transcription, but through another pathway independent of the estrogen receptor. In these studies, effects of genistein on basal and stimulated progesterone production were only observed at concentrations around 10^{-6} to 10^{-5} M.

Apigenin had completely opposite effects on steroidogenesis, stimulating both basal and FSH-induced progesterone production. This was observed at a dose 100 times lower than the dose of genistein required to induce inhibition, despite the fact that its relative binding affinity for the ER and transcriptional activity is higher (4, 6). This suggests that their actions on steroidogenesis are not mediated through a common ER. Indeed, since other kinase inhibitors such as lavendustin A (13), tyrphostin 23 (RG-50810), and H-89 (M. Lacey and S. Whitehead, unpublished data) can mimic the effects of genistein, it is likely that the inhibitory action of genistein is mediated through kinase inhibition rather than interactions with the ER.

Basal progesterone secretion was also stimulated by DDT and 4-octyl-phenol, although 10 times the dose of octyl-phenol was required before a significant stimulation was observed compared with DDT. Like apigenin, these stimulatory effects were reduced at high doses and though there was a loss of cell viability at the highest (100 μM) dose of apigenin and 4-octyl phenol, reduced responses were observed at doses where there was no apparent loss of cell viability (Table I).

Despite the stimulatory effects of DDT on basal progesterone production, it had no effect on FSH-induced production, while the response was enhanced in the presence of octyl-phenol. It has been shown that the relative binding affinities of octyl-phenol and DDT at the ERs are similar, but much lower than that of genistein (6). In contrast, it has been reported that up to 1000 times higher doses of DDT are required to induce significant transcriptional activity compared with octyl-phenol and this was through interaction with the ERα and not the ERβ. Octyl-phenol induced transcription by binding to both types of ERs (6).

A comparison of the reported relative binding affinities and transcriptional activities of these various xenoestrogens clearly bears no relation to the current observations with regard to the doses required and the effects they produced on progesterone production in granulosa-luteal cells. Furthermore, with the exception of genistein, estradiol did not mimic any of their effects. Why estrogen was inhibitory has not been defined, but there could be some feedback inhibition on the steroidogenic pathway.

In the rat ovary, estrogen is important in follicular development and enhances FSH-stimulated gene expression, and recent evidence shows the ERβ is the predominant receptor expressed in granulosa cells (22). While there has been some debate as to whether ERβ is expressed in corpora lutea of immature rats primed with pregnant mares' serum gonadotrophin (PMSG) or estrogen and treated with hCG (22, 23), to what extent this model resembles the untreated cyclic adult rats has not been determined. The reason for using granulosa-luteal cells in the present experiments, however, is because, unlike granulosa cells obtained from pre-ovulatory follicles, they produce relatively high concentrations of basal progesterone secretion in culture so that inhibitory effects of drugs can be readily observed. Interestingly, they are also more responsive to FSH than LH or hCG.

Table I. Cell Viability of Granulosa Luteal Cells after a 48-hr Culture with Endocrine Disrupting Chemicals (EDCs)

EDC	Con	Dose (μM)			
		1.0	10	50	100
Apigenin	0.040 (0.011)	0.055 (0.019)	0.037 (0.008)	0.033 (0.008)	0.016* (0.006)
Genistein	0.062 (0.010)	–	0.054 (0.009)	0.048 (0.013)	–
Coumarin	0.043 (0.007)	0.078 (0.015)	0.070 (0.012)	0.073 (0.012)	0.037 (0.008)
2,4′ DDT	0.031 (0.002)	0.062 (0.011)	0.052 (0.020)	0.029 (0.040)	0.037 (0.009)
4-Octyl-phenol	0.047 (0.008)	0.061 (0.011)	0.051 (0.016)	0.042 (0.012)	0.014* (0.003)

Note. Numbers are the optical density measurements (570–630 nm) obtained from culture wells after the cells had been exposed to MTT. Data represent the means (±SEM) from triplicate wells obtained from at least three separate experiments. Within each group there were no significant differences (Gabriel's tests) but $^*P < 0.05$ compared with the control value (unpaired Student's *t* test).

This study was not designed to investigate the cellular mechanisms of xenoestrogens, but to compare the effects of different endocrine-disrupting chemicals and estradiol on steroidogenesis. Reduced gonadal steroidogenesis has been reported in alligators obtained from contaminated lakes (24) and in fish living downstream of pulp mills (25). In controlled laboratory studies, genistein has been shown to inhibit FSH-induced progesterone synthesis in rat granulosa cells (13, 26) and a variety of phytoestrogens were reported to inhibit the conversion of estrone to estradiol, which is catalysed by 17β-hydroxysteroid oxidoreductase type1 (27). Hydroxylated polychlorinated biphenyls were reported to be potent inhibitors of estrogen sulfotransferase, thus indirectly increasing estradiol bioactivity (28).

Genistein and another phytoestrogen, diadzein, have been reported to suppress cortisol synthesis (29), while bisphenol A and 4-octyl-phenols, as well as estradiol and diethylstilboestrol inhibited hCG-stimulated progesterone production in a mouse Leydig tumor cell line (14). In contrast, another study reported that octyl-phenol had a biphasic effect on testosterone synthesis in Leydig cells from neonatal rats (30), similar to that on progesterone synthesis reported here. A metabolite of the common pesticide, methoxychlor, was shown to reduce testosterone production by inhibiting the expression of the cholesterol side-chain cleavage enzyme (31).

Overall, the evidence to date is that chemicals that are known to bind to the ER appear to exert inhibitory effects on steroidogenesis. The current difficulty, however, in investigating endocrine-disrupting chemicals is that most, if not all, compounds and/or their metabolites have multiple sites of action. For example, different derivatives of coumarin can inhibit tyrosine kinase activity (32) or inhibit steroid sulphatase (33) and DDT can induce the expression of hepatic cytochrome P_{450} (CYP) genes (34). It is not pertinent to discuss all the potential actions of the varied xenoestrogens, but one property many of them share, and including estradiol itself, is antioxidant activity (35, 36). This is significant because steroid biosynthesis is accompanied by the formation of oxygen radicals that can affect the cyclical steroidogenic and morphogenic changes in ovarian follicles. A recent study, however, showed that the effects of octyl-phenol on testosterone production in Leydig cells was independent of pro- or antioxidant properties (30).

The present report shows that different xenoestrogens have very different effects on basal and FSH-stimulated progesterone synthesis. Thus, the cause-and-effect relationship of the xenoestrogens tested is dissimilar and may not be related to their ability to bind to ERs and initiate estrogen-activated transcription. Indeed, this was demonstrated in a recent report that showed that the profile of uterotropic responses to different phyto- and xenoestrogens did not always match the induction of estrogen sensitive genes in the uterus (37).

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