

# Phytoestrogens inhibit aromatase but not 17 $\beta$ -hydroxysteroid dehydrogenase (HSD) type 1 in human granulosa-luteal cells: evidence for FSH induction of 17 $\beta$ -HSD

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**BACKGROUND:** Studies using purified enzyme preparations, placental microsomes or cell lines have shown that certain phytoestrogens can inhibit the enzymes that convert androgens to estrogens, namely aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase (HSD) type 1 and type 5. The study aim was to investigate the effects of selected phytoestrogens on aromatase and 17 $\beta$ -HSD type 1 activity in primary cultures of human granulosa-luteal (GL) cells. **METHODS AND RESULTS:** GL cells, cultured for 48 h in medium containing 5% fetal calf serum and for a further 24 h in serum-free medium with or without hFSH or hCG, were exposed to steroid substrates during the last 1–4 h of the experiment. The production of progesterone in the presence of pregnenolone or estradiol synthesis from androstenedione, estrone or testosterone showed dose- and time-dependent increases. Whilst hCG priming had no effect on progesterone production, FSH priming induced mean 68 and 56% increases in the production of estradiol from androstenedione (A-dione) and estrone respectively, but had no significant effect on the metabolism of testosterone to estradiol. None of the phytoestrogens investigated had any acute effects on enzyme activity. In contrast, when GL cells were exposed to the compounds for 24 h prior to exposure to steroid substrates for 4 h, 10  $\mu$ mol/l apigenin and zearalenone significantly inhibited aromatase activity, whilst biochanin A and quercetin had no effect. None of the phytoestrogens inhibited FSH-induced 17 $\beta$ -HSD type 1 activity, and only quercetin significantly inhibited progesterone production. **CONCLUSIONS:** The inability of phytoestrogens to acutely inhibit steroidogenic enzymes in human GL cells (as has been shown in cell-free models) suggests that they are either rapidly metabolized to relatively inactive compounds or that the high enzyme activity in human GL cells masks any inhibitory effects of the compounds at the concentration tested.

*Key words:* aromatase/human granulosa luteal cells/4 hydroxy-androstenedione/17 $\beta$  hydroxysteroid dehydrogenase/phytoestrogens

## Introduction

Phytoestrogens are plant-derived, non-steroidal compounds that bind to the estrogen receptor (ER) and can induce estrogen-dependent gene transcription (Kuiper *et al.*, 1998). They are diphenolic substances with structural similarity to estrogen, and can be divided into three main classes: flavanoids; coumestans; and lignans. The group of flavanoids includes the flavanones, flavones and isoflavones, and these are found in almost all plant families in leaves, stems, roots, flowers and seeds (Mazur and Adlercreutz, 2000). Most flavanoids in plants are present as glycosides but they are hydrolysed in the human gastrointestinal tract to biologically active aglycones (Setchell, 1998).

Whilst relatively little is known about the absorption, metabolism and bioavailability of various phytoestrogens, on a high soy diet or a vegetarian diet, mean plasma concentra-

tions of the various phytoestrogens that have been measured can range from 100 nmol/l to 1  $\mu$ mol/l (Mazur and Adlercreutz, 2000). These are concentrations at which phytoestrogens have been shown to have estrogenic effects *in vitro* (Zava and Duwe, 1997; Maggiolini *et al.*, 2001). In this respect, soy is being heavily promoted as a natural alternative to hormone replacement therapy.

There is, however, a paradox. Both epidemiological studies (Aldercreutz, 1998; Barnes, 1998) and laboratory investigations (Barnes, 1997) have suggested that dietary compounds may have a major protective effect against post-menopausal breast cancer. Thus, the markedly reduced incidence of breast cancer seen amongst many Asian populations compared with women in Northern Europe and America has been attributed to their diets that are rich in soy products (Aldercreutz, 1998). These contain high concentrations of conjugated isoflavones

that are thought to have a protective role in breast cancer by acting as weak estrogens or anti-estrogens by competing with endogenous estrogens for binding to the ER.

There is, however, increasing evidence that phytoestrogens may bind to aromatase and/or 17 $\beta$ -hydroxysteroid dehydrogenase (HSD) and thereby reduce the availability of these enzymes for the production of estrogen from androgen precursors and/or the production of estradiol from weak estrogens (Kao *et al.*, 1998; Mäkelä *et al.*, 1998; Le Bail *et al.*, 2000). In breast tissue of post-menopausal women these enzymes are responsible for the local production of estrogens from circulating adrenal androgens (Simpson *et al.*, 2000).

Previous studies investigating the effects of phytoestrogens on steroidogenic enzyme activity have typically been carried out on purified or recombinant enzyme preparations, placental microsomes or breast cancer cell lines (Mäkelä *et al.*, 1998; Le Bail *et al.*, 2000; Krazeisen *et al.*, 2001). These studies showed that the concentrations inducing a 50% inhibition of enzyme activity (IC<sub>50</sub>) of the most potent phytoestrogens investigated were generally between 1 and 20  $\mu$ mol/l. Thus, the purpose of the present study was to establish an experimental protocol that would allow an investigation of the effects of phytoestrogens on aromatase and 17 $\beta$ -HSD type 1 in primary cultures of human cells. The phytoestrogens investigated were two flavones (apigenin and quercetin), an isoflavone (biochanin A) and the mycotoxin, zearalenone. These compounds have been shown to have relatively potent effects on aromatase and/or 17 $\beta$ -HSD compared with other phytoestrogens (Le Bail *et al.*, 1998; Jeong *et al.*, 1999; Krazeisen *et al.*, 2001), although their reported potency varies according to the experimental model employed.

## Materials and methods

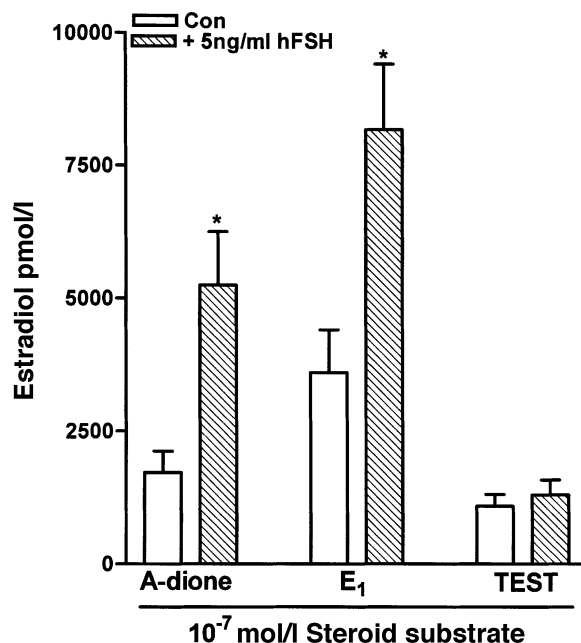
### Granulosa-luteal cells

Granulosa-luteal cells were obtained from the Assisted Conception Units at St George's Hospital and King's College Hospital, London, with signed consent of the patients. They were processed within 2 to 5 h of oocyte retrieval, depending on their source. After washing, the cells were purified on a 60% Ficoll gradient and cultured in a 250  $\mu$ l volume in 96-well culture plates. For the initial experiments, cells were cultured at a concentration of  $\sim 3 \times 10^5$  cells/ml (Figures 1 and 2) and subsequently at a lower concentration of  $10^5$  cells/ml. The culture medium was DMEM containing 2 mmol/l glutamate, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin (Sigma, Poole, Dorset, UK).

For the 'acute' experiments, cells were cultured for 48 h in medium supplemented with 5% fetal calf serum (FCS) and a further 24 h without FCS but with or without 5 ng/ml hFSH, as appropriate. Fresh serum-free medium plus steroid substrates with or without test compounds were added for the last 4 h of culture. This was the period during which estradiol or progesterone production was measured.

For the 'chronic' experiments, the protocol was identical except that the cells were exposed to phytoestrogens for 24 h prior to their 4 h exposure to steroid substrates with or without test compounds. For the time-course studies, 25  $\mu$ l of medium was taken at 1 and 2 h and the volume replaced with fresh medium. The dilution factor was taken into account in the analysis of the data.

At the end of the experiment, media samples were taken and stored at  $-20^\circ\text{C}$  and viability of cells was routinely tested either with the Trypan blue exclusion test or with Almar blue (Serotec, Oxford, UK)



**Figure 1.** Combined data of nine individual experiments with triplicate observations, showing the mean concentration of estradiol (E<sub>2</sub>) synthesized over a 4 h period using 10<sup>-7</sup> mol/l androstenedione (A-dione), estrone (E<sub>1</sub>) or testosterone (TEST) as substrates for steroid synthesis. Cells, at a concentration of 3 × 10<sup>5</sup> cells/ml were cultured for 48 h in the presence of 5% fetal calf serum, for 24 h in serum-free medium, either with (hatched bars) or without (open bars) 5 ng/ml hFSH, and for a final 4 h in fresh medium but in the presence of 10<sup>-7</sup> mol/l substrates as indicated. Data represent mean ± SEM. \*, P < 0.001 compared with paired control values without hFSH priming (Student's *t*-test).

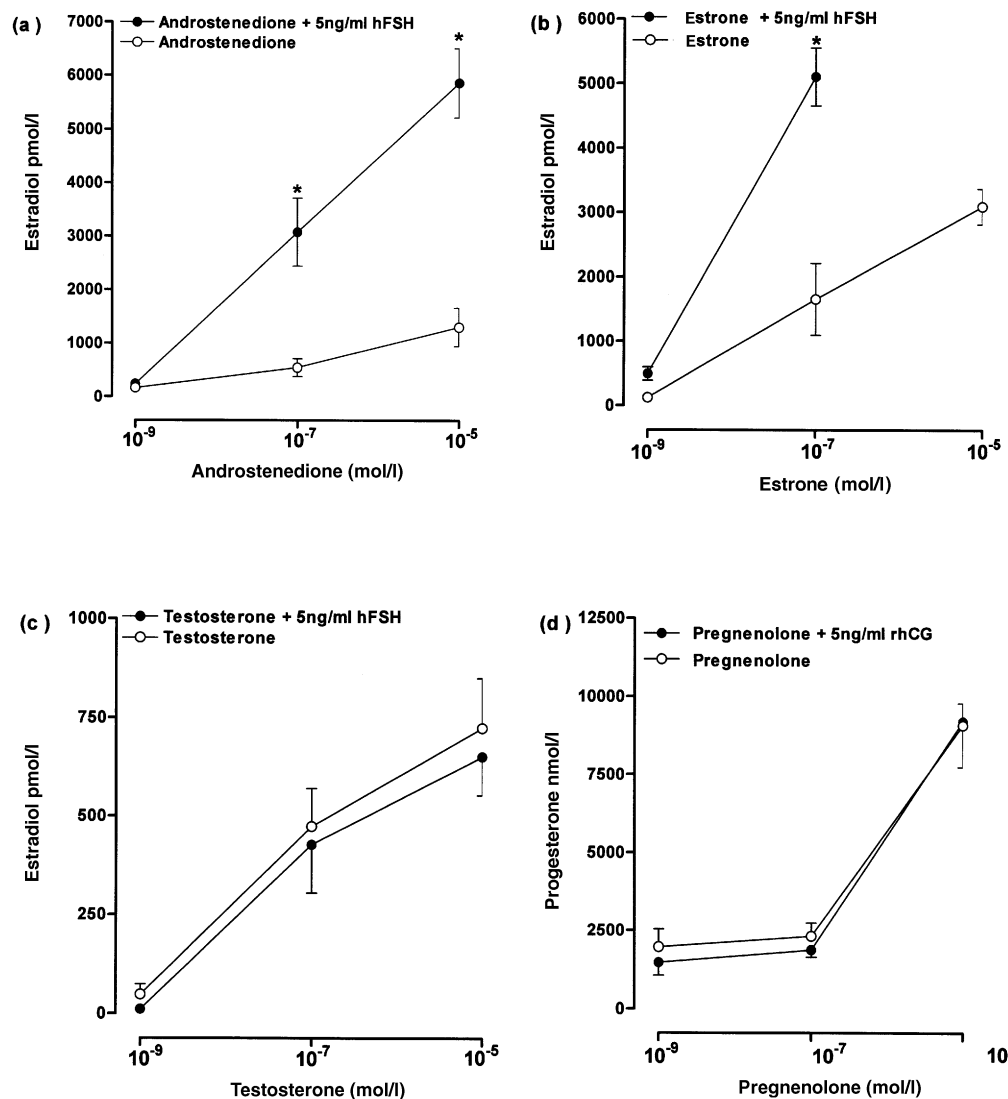
according to the manufacturer's instructions. This is a non-toxic REDOX indicator that provides a measure of mitochondrial activity and has been found to be more sensitive than the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, particularly when relatively small numbers of cells are being assayed.

### Drugs

The following drugs were used in these experiments: apigenin (4',5,7-trihydroxyflavone), biochanin A (4'-methoxy-5,7-dihydroxyisoflavone), quercetin (3,3', 4',5,7, pentahydroxyflavone), zearalenone, androstenedione, 4-hydroxyandrostene-3,17-dione (4OH-A), estrone, pregnenolone, testosterone, human FSH (hFSH) and recombinant hCG (rhCG). hFSH and rhCG were kindly supplied by The National Hormone and Pituitary Agency, Torrance, CA, USA; all other drugs were supplied by Sigma UK. All steroids were initially dissolved in ethanol and diluted appropriately with culture medium before being stored as stock solutions. Stock solutions were then further diluted with medium for each experiment and added to cultures in 10  $\mu$ l volumes to give the desired final concentration. Controls were performed to ensure that the maximum volume of ethanol diluent (1  $\mu$ l) did not affect cellular responses.

### Steroid assays

Progesterone and estradiol samples were measured in duplicate using a direct radioimmunoassay (RIA) kit (ICN Pharmaceuticals Ltd, Basingstoke, UK) according to the manufacturer's instructions. All drugs used in these experiments were tested for their possible cross-reactivity with the anti-sera used, but none was detected. The cross-



**Figure 2.** Estradiol ( $E_2$ ) and progesterone concentrations in the culture medium after a 4 h incubation with (a) testosterone, (b) androstenedione, (c) estrone and (d) pregnenolone. Cells, at a concentration of  $3 \times 10^5$  cells/ml were cultured for 48 h in the presence of 5% fetal calf serum, for 24 h in serum-free medium, with or without 5 ng/ml hFSH, and for a final 4 h in fresh medium but in the presence of  $10^{-9}$  to  $10^{-5}$  substrates as indicated. Data represent mean  $\pm$  SEM of three individual experiments each with triplicate wells. \*,  $P < 0.001$  compared with the corresponding values in the absence of FSH (Student's  $t$ -test).

reactivity of the progesterone anti-serum with  $20\alpha$ -dihydroprogesterone,  $17\alpha$ -hydroxyprogesterone and pregnenolone was 5.4, 0.6 and 0.4% respectively, and that with the estradiol anti-serum with estrone, estriol, progesterone and testosterone and androstenedione was 20, 1.5,  $<0.01$ ,  $<0.01$  and  $<0.01\%$  respectively. Inter- and intra-assay coefficients of variation were respectively 6.2 and 3.0% for the progesterone assay, and 9.1 and 5.2% for the estrogen assay. For each experiment, the cross-reactivity of estrone with the estradiol antibody was monitored by incubating the appropriate concentration of estrone in culture medium without GL cells for 4 h. The mean estradiol concentration measured in the 'blank' wells was subtracted from the appropriate experimental measurements. When  $10 \mu\text{mol/l}$  estrone was added to the cultures the cross-reactivity was  $>20\%$  and thus these results were excluded.

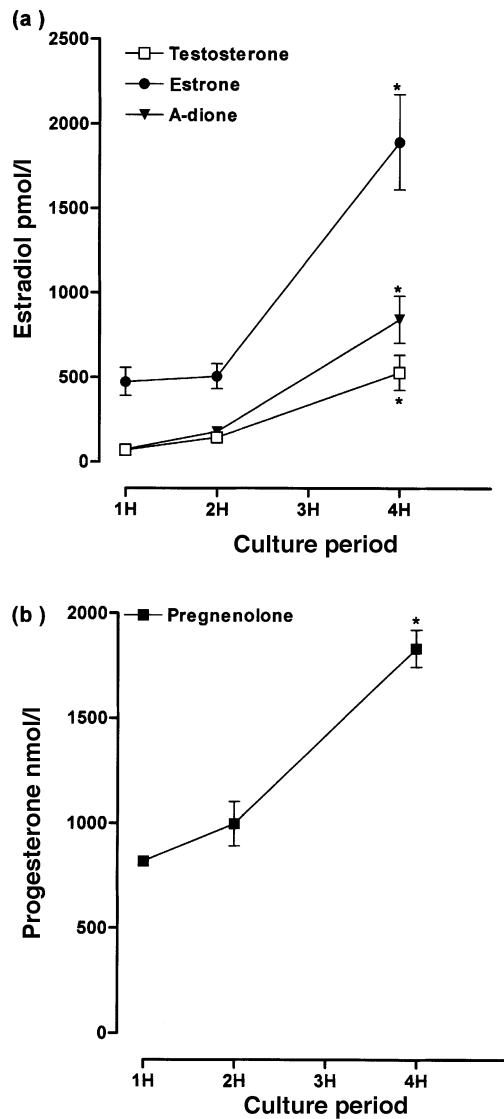
#### Statistical analysis

Data shown represent mean  $\pm$  SEM of triplicate cultures obtained from at least three independent experiments, and  $n$  = the total number

of individual observations. When comparing several groups of data, statistical differences were obtained with an analysis of variance, followed by Gabriel's test. A Student's  $t$ -test was used to test significance between two groups of data.

#### Results

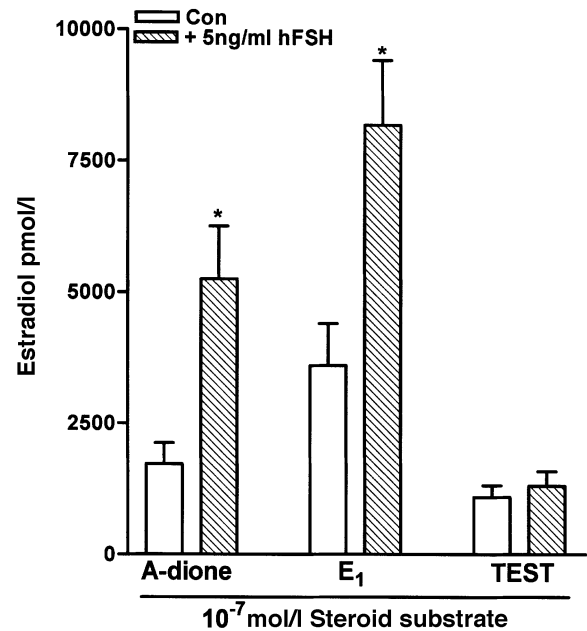
The combined results of 12 individual experiments showed that FSH priming significantly increased the synthesis of estradiol from androstenedione and estrone, but had no effect on estradiol synthesis when testosterone was used as a substrate (Figure 1) In the absence of a steroid substrate, basal estradiol production over a 4 h period was either undetectable or was  $<50$  pmol/l compared with progesterone production that was between 350 and 1300 nmol/l. In three individual experiments, the production of estradiol in the presence of increasing concentrations of the three substrates showed a linear dose-



**Figure 3.** Time course of the conversion of testosterone, estrone and androstenedione on (a) estradiol ( $E_2$ ) and (b) progesterone production in GL cells cultured at a concentration of  $10^5$  cells/ml for 48 h in the presence of fetal calf serum and 24 h in serum-free medium but in the presence of 5 ng/ml hFSH (a) or 1 ng/ml rhCG (b). Fresh medium plus steroids were added for the final 4 h of the experiment. Data represent the mean  $\pm$  SEM of three individual experiments (in triplicate); \*,  $P < 0.01$  compared with the control, value (Student's *t*-test).

dependent relationship (Figure 2a–c). This was not observed with progesterone production in the presence of pregnenolone, and only the highest concentration of pregnenolone 10  $\mu$ mol/l significantly increased progesterone production (Figure 2d). Priming with rhCG had no effect on progesterone synthesis.

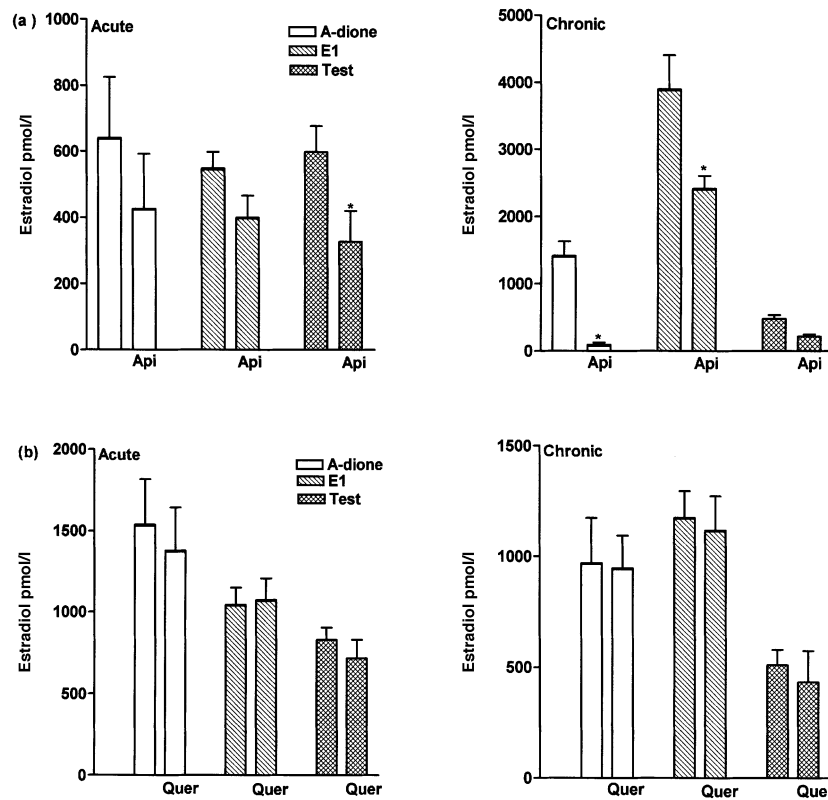
The time course of the conversion of the various substrates showed that between 1 and 2 h there was no marked increase in either estradiol or progesterone production, but between 2 and 4 h there was a marked and significant increase in steroid production (Figure 3a and b). It was thus established that a 24-h exposure to FSH followed by a 4-h exposure to steroid substrates at a dose of 100 nmol/l was a suitable protocol for investigating estradiol production.



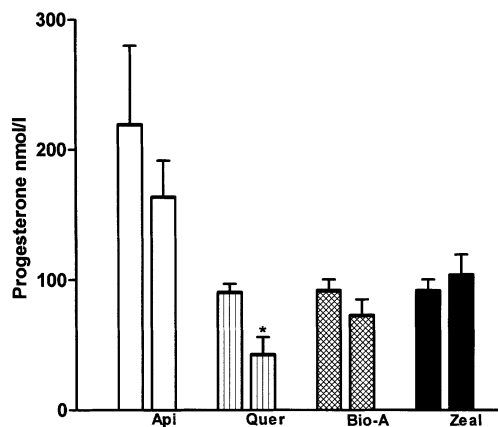
**Figure 4.** Effects of the aromatase inhibitor, 4-hydroxyandrostenedione (4OH-A), on the synthesis of estradiol ( $E_2$ ) from  $10^{-7}$  mol/l androstenedione (A-dione) estrone ( $E_1$ ) or testosterone (TEST). GL cells, cultured at a concentration of  $10^5$  cells/ml, were primed with 5 ng/ml hFSH for 24 h prior to the addition of fresh medium and steroid substrates for 4 h with or without 10  $\mu$ mol/l 4OH-A. Data represent mean  $\pm$  SEM of three to four individual experiments (triplicate observations); \*,  $P < 0.01$  compared with control (Con) values in the absence of 4OH-A (Student's *t*-test).

To test the assumption that the experimental protocol was providing data on the activity of steroidogenic enzymes, the effects of a well-established aromatase inhibitor 4OH-A were investigated (Figure 4). This showed a selective and significant inhibition of estradiol production from either androstenedione or testosterone, both of which require aromatase. In contrast, 4OH-A did not inhibit the conversion of estrone to estradiol that only requires  $17\beta$ -HSD type 1 activity and, interestingly, there was a modest, but significant, increase in estradiol production in the presence of this aromatase inhibitor.

Apigenin significantly inhibited estradiol production from testosterone, and whilst the mean concentration of estradiol produced from the metabolism of either estrone or androstenedione were reduced, results were not statistically significant (Figure 5a). A different picture was seen when GL cells were exposed to apigenin for 24 h prior to testing enzyme activity. In this case, estradiol synthesis was markedly reduced irrespective of the steroid substrate (Figure 5a). The other flavone, quercetin, had no significant effects on estradiol production from any of the three substrates, irrespective of whether cells were exposed either acutely (4 h) or chronically (24 h) to these phytoestrogens (Figure 5b). However, unlike the other phytoestrogens investigated, quercetin significantly inhibited progesterone synthesis (Figure 6). Exposure of GL cells to the isoflavone biochanin A for 24 h prior to testing enzyme activity reduced the mean concentration of estradiol produced from all three substrates, but results were not significant (Figure 7a).



**Figure 5.** Acute and chronic effects of apigenin (a) and quercetin (b) on the synthesis of estradiol ( $E_2$ ) from 100 nmol/l androstenedione (A-dione), estrone ( $E_1$ ) or testosterone (TEST). For the acute experiments, GL cells, cultured at a concentration of  $10^5$  cells/ml, were primed with 5 ng/ml hFSH for 24 h prior to the addition of fresh medium and steroid substrates for 4 h with or without 10  $\mu$ mol/l apigenin or quercetin. For the chronic experiments, cells were exposed to FSH and the phytoestrogens for 24 h prior to the addition of fresh medium, compounds and steroid substrates for the last 4 h. Data represent mean  $\pm$  SEM of three to four individual experiments (triplicate observations); \*,  $P < 0.01$  compared with control (Con) values in the absence of apigenin (Student's  $t$ -test).



**Figure 6.** Chronic effects of apigenin (Api), quercetin (Quer), biochanin A (Bio-A) and zearalenone (Zeal) on progesterone production. GL cells, at a concentration of  $10^5$  cells/ml were cultured for 48 h in the presence of 5% fetal calf serum and then exposed to phytoestrogens for 24 h prior to the addition of 100 nmol/l pregnenolone plus phytoestrogens in fresh medium. Samples were collected after 4 h. Data represent mean  $\pm$  SEM of three to four individual experiments (triplicate observations); \*,  $P < 0.01$  compared with control (Con) values in the absence of quercetin (Student's  $t$ -test).

Exposure of GL cells to zearalenone for 24 h induced a marked reduction in aromatase activity as evidenced by significant reductions in estradiol production from androstenedione and

**Table I.** Viable cell mass<sup>a</sup> measured with Almar blue after granulosa-luteal (GL) cells were cultured with  $10^{-5}$  mol/l phytoestrogens for 24 h

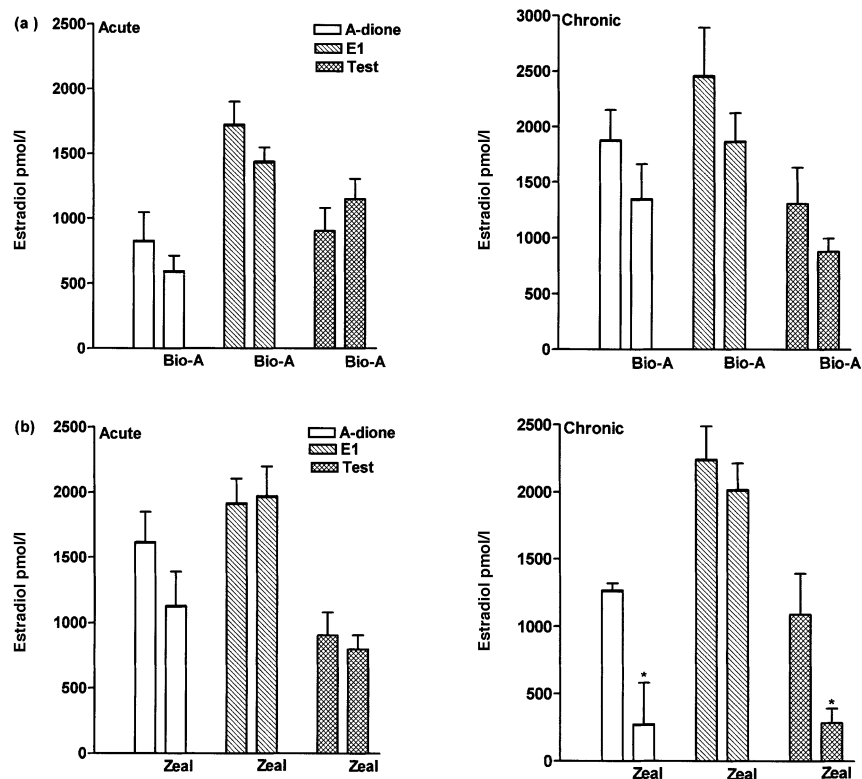
	Androstenedione	Estrone	Testosterone
Control	0.66 $\pm$ 0.05	0.71 $\pm$ 0.04	0.63 $\pm$ 0.03
Apigenin	0.71 $\pm$ 0.04	0.69 $\pm$ 0.04	0.66 $\pm$ 0.02
Control	0.50 $\pm$ 0.03	0.58 $\pm$ 0.03	0.53 $\pm$ 0.07
Quercetin	0.55 $\pm$ 0.07	0.61 $\pm$ 0.06	0.59 $\pm$ 0.06
Control	0.49 $\pm$ 0.10	0.45 $\pm$ 0.06	0.48 $\pm$ 0.08
Biochanin A	0.51 $\pm$ 0.03	0.43 $\pm$ 0.06	0.43 $\pm$ 0.06
Control	0.49 $\pm$ 0.08	0.56 $\pm$ 0.05	0.55 $\pm$ 0.06
Zearalenone	0.50 $\pm$ 0.08	0.53 $\pm$ 0.07	0.52 $\pm$ 0.06

<sup>a</sup>Values are the mean  $\pm$  SEM optical density measurements (570–600 nm) of three to four independent experiments of triplicate observations.

testosterone but without effect the production of estradiol from estrone (Figure 7b). Viability studies run routinely at the end of every chronic experiment showed that none of the phytoestrogens reduced cell viability (Table I).

## Discussion

The results of the present study have shown that there is a dose- and time-dependent conversion of androstenedione, testosterone and estrone to estradiol over a 4 h period, and that the aromatase inhibitor, 4OH-A (Devoto *et al.*, 1991) inhibited the production of estradiol from androstenedione and testosterone



**Figure 7.** Acute and chronic effects of biochanin A (a) and zearalenone (b) on the synthesis of estradiol ( $E_2$ ) from different steroid substrates. For details, see legend of Figure 5.

as predicted. The GL-cell conversion of testosterone to estradiol was inhibited by a mean of 62%, whilst the conversion of androstenedione to estradiol was inhibited by a mean of 88%. There is no apparent reason why 4OH-A should be more potent in inhibiting the metabolism of A-dione to estradiol compared with testosterone to estradiol, nor why the conversion of estrone to estradiol should be significantly enhanced in the presence of 4OH-A. There could be some product inhibition or local feedback regulation of enzyme activity. In this respect, it is interesting to note that low concentrations of estradiol can dose-dependently inhibit the conversion of estrone sulphate to estradiol in MCF-7 breast cancer cells (Pasqualini and Chetrite, 2001).

Several previous studies have shown that GL cells obtained from natural cycles are sensitive to FSH, but that those obtained from stimulated cycles are insensitive (Bergh *et al.*, 1991; Mason *et al.*, 1993; Lambert *et al.*, 1995; Foldesi *et al.*, 1998). It has been reported that a long pre-incubation period of 72 h followed by exposure to FSH for 4–6 days increased basal estradiol production in GL cells in the absence of any androgenic substrate, but in the presence of testosterone FSH had no effect on estradiol production. More recently, it has been reported that GL cells exposed to FSH for 24 h dose-dependently increased estradiol synthesis from testosterone over a 2 h period (Lambert *et al.*, 2000). In the present study, using a protocol similar to that used by Lambert and colleagues, no increase in aromatase activity (as assessed by the conversion of testosterone to estradiol) was observed. In contrast, however, FSH priming significantly increased the

production of estradiol from both estrone and androstenedione. The former requires  $17\beta$ -HSD type 1 activity and the latter aromatase and  $17\beta$ -HSD type 1 if the conversion is via estrone, or  $17\beta$ -HSD type 5 and aromatase if the conversion is via testosterone. Both type 1 and type 5  $17\beta$ -HSD are present in the human ovary (Labrie *et al.*, 2000).

Others (Ghersevich *et al.*, 1994a) have reported that in rat granulosa cells, rFSH stimulated expression and activity of  $17\beta$ -HSD type 1, and subsequently it was shown that FSH induction of this enzyme was through a protein kinase A (PKA)-dependent pathway (Kaminiski *et al.*, 1997). In cultured human granulosa-luteal cells, gonadotrophins were shown to have no effect on  $17\beta$ -HSD, although aromatase expression and activity was increased (Ghersevich *et al.*, 1994b). Interestingly, there is evidence of an ovarian deficiency of the enzyme(s) in polycystic ovary syndrome (Toscano *et al.*, 1990).

It has generally been assumed that the action of aromatase on the conversion of testosterone to estradiol has been the major pathway through which estrogens are produced by granulosa cells. Recent studies have shown, however, that human type 5  $17\beta$ -HSD, which converts androstenedione to testosterone, is expressed exclusively in thecal cells (Luu-The *et al.*, 2001). It was suggested that the production of estradiol in ovarian granulosa cells is preferentially via aromatization of 4-androstenedione to estrone and a subsequent conversion of estrone to estradiol by type 1  $17\beta$ -HSD. This would be in agreement with the higher affinity of aromatase for androstenedione compared with testosterone (Luu-The *et al.*, 2001).

The present studies certainly agree with this hypothesis, as the production of estradiol from precursors that involved the activity of type 1 17 $\beta$ -HSD generally produced higher concentrations of estradiol than testosterone alone (Figure 1). They also show that 17 $\beta$ -HSD type 1 in human GL cells can be modulated by FSH rather than aromatase, since the mean increase in estradiol production after FSH priming compared with control values was 16, 56 and 68% for testosterone, estrone and androstenedione respectively. This is consistent with the observations that FSH induction of aromatase is not generally observed in GL cells (see Lambert *et al.*, 2000). Whether or not type 5 17 $\beta$ -HSD is also modulated by FSH can neither be supported nor negated by this study.

Previous studies investigating the inhibitory effect of phytoestrogens on 17 $\beta$ -HSD or aromatase activity in placental microsomes or partially purified or recombinant enzyme preparations show that the most potent phytoestrogens induce 50% inhibition of enzyme activity (IC<sub>50</sub>) at concentrations in the range of 0.5 to 20  $\mu$ mol/l. Thus, for these initial studies to screen the effects of phytoestrogens, a relatively high dose of 10  $\mu$ mol/l was used. This is the same concentration of 4OH-A used to inhibit aromatase activity by competitively binding to the enzyme.

In human GL cells, 10  $\mu$ mol/l apigenin had only weak inhibitory effects on enzyme activity, although after cells had been exposed to this phytoestrogen for 24 h there was a marked loss of both aromatase and 17 $\beta$ -HSD type 1 activity. In contrast, the other flavone, quercetin was without effect on either enzyme. It has been reported (Mäkelä *et al.*, 1995, 1998) that 1.2  $\mu$ mol/l apigenin inhibited the conversion of tritiated estrone to estradiol by ~40% and 80% in a purified 17 $\beta$ -HSD type 1 preparation and in T-47D breast cancer cells respectively, whilst quercetin at the same dose had no effect in either preparation. In human placental microsomes, apigenin was similarly shown to be a relatively potent inhibitor of 17 $\beta$ -HSD type 1 activity, with an IC<sub>50</sub> of 0.3  $\mu$ mol/l (Le Bail *et al.*, 1998). Others (Krazeisen *et al.*, 2001) showed that apigenin and quercetin were the most potent flavones inhibiting the reductive activity of recombinant 17 $\beta$ -HSD type 5, with IC<sub>50</sub> values in the order of 20  $\mu$ mol/l. With regard to the effects of flavones on aromatase activity, apigenin has been reported to inhibit this enzyme, with an IC<sub>50</sub> of 2.9  $\mu$ mol/l, in placental microsomes (Le Bail *et al.*, 1998) and ~5  $\mu$ mol/l in partially purified placental aromatase (Jeong *et al.*, 1999). Both apigenin and quercetin were found to be potent inhibitors of the aromatase enzyme complex from rainbow trout (Pelissero *et al.*, 1996), and quercetin was also found to inhibit aromatase activity in colorectal cancer cell lines (Fiorelli *et al.*, 1999).

Like apigenin, the isoflavone genistein has also been reported to inhibit 17 $\beta$ -HSD type 1 activity (Le Bail *et al.*, 1998; Whitehead *et al.*, 2002), whilst the effects of genistein and other isoflavones on aromatase inhibition have generally been reported to be either weak or undetectable (Kao *et al.*, 1998; Le Bail *et al.*, 1998; Whitehead *et al.*, 2002).

In the present study, it was found that another isoflavone, biochanin A, had no significant effects on either 17 $\beta$ -HSD type 1 or aromatase, although others (Le Bail *et al.*, 2000) reported that biochanin A inhibited human placental aromatase, 17 $\beta$ -

HSD and 3 $\beta$ -HSD activities with IC<sub>50</sub>s of 49, 4.9 and 10  $\mu$ mol/l respectively. Current investigations include the examination of dose–response curves to several phytoestrogens, and indeed some—including biochanin A—do show a weak dose-related inhibition of aromatase in human GL cells. Compared with controls, however, significance is only reached at a ten times higher dose (100  $\mu$ mol/l) than those used in the present series of experiments.

The mycotoxin, zearalenone, showed potent effects on inhibiting the production of estradiol from androstenedione and testosterone, but had no effect on 17 $\beta$ -HSD type 1 activity converting estrone to estradiol. The latter result agrees with those of others (Mäkelä *et al.*, 1995) on purified 17 $\beta$ -HSD type 1. Interestingly, it has also been found (Krazeisen *et al.*, 2001) that the five most potent phytoestrogens inhibiting the reductive activity of 17 $\beta$ -HSD type 5 were zearalenone, coumestrol, quercetin, biochanin A and apigenin, with IC<sub>50</sub>s of 4, 5, 9, 14 and 20  $\mu$ mol/l respectively.

The effects of the phytoestrogens investigated in the present study were not always in agreement with studies concerned with their effects on cell-free systems, or indeed with cancer cell lines (Mäkelä *et al.*, 1998; Fiorelli *et al.*, 1999). One explanation is that the enzyme concentration/activity is very high in primary cultures of GL cells. Indeed, preliminary studies in our laboratory on MCF-7 cells have found that the production of estradiol/75 000 cells from the various substrates is five to ten times lower after a 48 h period compared with that of human GL cells after a 4 h period.

In conclusion, these studies have established an experimental protocol for investigating the effects of phytoestrogens on enzyme activity in primary cultures of GL cells, and have shown that FSH priming—in line with other studies—did not induce aromatase in GL cells but did induce 17 $\beta$ -HSD type 1. In contrast with other studies there was no acute effect of phytoestrogens on enzyme activity but, when cells had been exposed to the compounds for 24 h and then exposed to steroid substrates for 4 h, inhibition of aromatase activity was observed. This suggests either that the lipid solubility of phytoestrogens is low and so permeation into the cells is slow, or that the compounds are being metabolized to inactive substances and/or the compounds and their metabolites are altering the expression, and hence concentration, of intracellular steroidogenic enzymes.

### Acknowledgements

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