Regulation of epidermal growth factor receptor by androgens in human endometrial cells in culture

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Women with polycystic ovaries (PCO) have a thicker endometrium than women with normal ovaries. This cannot be due to unopposed oestrogen, as it occurs in ovulatory cycles. Androgens may be involved, as these are raised in women with PCO. The endometrium was enzymatically dispersed and glands and stromal cells separated. Cells were incubated in Ham’s F10 medium supplemented with 5% charcoal-stripped fetal calf serum and either androgens or vehicle. Specific binding of [125I]-labelled EGF was measured. Testosterone and dihydrotestosterone (DHT) (10 µmol/l) increased EGF receptor concentration (control 100 ± 9%, testosterone 196 ± 23% control; control 100 ± 1%, DHT 244 ± 6% control) but did not alter receptor affinity. The effect of testosterone was inhibited by the anti-androgen hydroxyflutamide, but not by the anti-oestrogen ICI182780 nor the aromatase inhibitor 4-hydroxyandrostenedione. EGF receptor levels were increased by androstenediol (control 100 ± 2%, androstenediol 120 ± 10% control) but not by androstenedione, dehydroepiandrosterone (DHA), DHA sulphate or androstenedione. Testosterone and DHT increased EGF receptor concentrations in glandular epithelium (control 100 ± 24%, testosterone 147 ± 5%, DHT 185 ± 30% control). These data suggest that androgens may have an effect on the endometrium via an increase in EGF receptor concentrations.

Key words: androgen/EGF/endometrium/human/receptor

Introduction

Of the female population, 22% have polycystic ovaries (PCO) (Polson et al., 1988). Definitive diagnosis is by pelvic ultrasonography, the criteria including bilateral ovarian enlargement, 10 or more follicles with a peripheral distribution, and increased density and area of stroma (Adams et al., 1986). Although the endocrine and clinical presentations are heterogeneous, the most consistent biochemical abnormality in women with PCO is hyperandrogenaemia (Franks, 1991). Androgens are raised in those women with PCO who are anovulatory and those who are ovulating. They are also raised in women with recurrent miscarriage (Watson et al., 1993), of whom >50% have PCO (Clifford et al., 1994). A study by Tulppala et al. (1993) concluded that although the presence of PCO did not predict miscarriage, those patients who miscarried had higher levels of total testosterone, free testosterone and dehydroepiandrosterone sulphate (DHAS) than those with continuing pregnancies.

The endometrium of women with PCO is thicker than that of women with normal ovaries (Adams et al., 1988). This cannot be explained solely on the basis of prolonged exposure to oestrogen unopposed by progesterone, since the endometrium in the follicular phase of women with PCO who are ovulating is thicker than that of women with normal ovaries. Moreover, PCO is associated with an increased incidence of endometrial cancer (Sherman and Brown, 1979) and accordingly, postmenopausal women with endometrial cancer have an increased ovarian secretion of androstenedione and testosterone (Nagamani et al., 1986). Vitoratos et al. (1990) found that women with adenomatous hyperplasia of the endometrium had significantly higher peripheral venous concentrations of androgens than women with normally proliferating endometrium. However, although the women were pre-menopausal, neither cycle history nor ovarian morphology were noted. The role of androgens in endometrial growth and development is at present unclear. Studies of the rat uterus in vivo have demonstrated a stimulation of growth by androgens, and histological differences in androgen-stimulated rat uteri have been observed (Datta et al., 1974). However, the growth of human endometrial cells in culture is inhibited by androgens (Neulen et al., 1987; Rose et al., 1988), and suppression of endometriosis is achieved in vivo by the administration of androgenic drugs such as danazol. In a study by Narukawa et al. (1994) testosterone increased the production of prolactin by endometrial cells, suggesting that androgens may be involved in the mechanism of decidualization.

The interactions between growth factors and steroids in reproductive tissues are well established. The actions of oestrogen and progesterone in uterine and endometrial development and differentiation are mediated by epidermal growth factor (EGF) (DiAugustine et al., 1988) and its receptor (Mukku and Stancel, 1985; Taketani and Mizuno, 1991). In the prostate gland, the proliferative effect of testosterone is mediated by the induction of the EGF receptor (Liu et al., 1993). The aim of the present study was to investigate the effects of androgens on EGF receptor in endometrial stromal cells and glands.

Materials and methods

Materials

Culture media, trypsin/EDTA, fetal calf serum and ‘Linbro’ multiwell plates were obtained from Flow Laboratories Ltd, Rickmansworth.
Preparation and treatment of cell cultures

Stromal cells and intact glands were isolated by enzymatic dispersion with 0.125% w/v collagenase (Type XI) (Bonney et al., 1991). Stromal cells were isolated and plated at a density of 2×10⁵ cells per well, directly into 24-well multiwell plates (well diameter 16 mm). Glands were distributed evenly between half the number of wells required for stromal cells. Steroid or vehicle (ethanol) alone were added to 0.5 ml Ham’s F10 supplemented medium such that the final volume of ethanol was 0.1%. Blood cells and debris were removed after 24 h by washing three times with Ham’s F10 medium supplemented with kanamycin and nystatin, leaving the stromal cells attached to the surface of the well. The medium and treatments were replenished daily for 5 days. The cells were washed three times with Ham’s F10 medium containing only kanamycin and nystatin on the sixth day, and then incubated with steroid (or ethanol) for a further 24 h in Ham’s F10 medium without serum but supplemented with 10 mmol glutamine/l, 10 mg insulin/ml, 100 ng hydrocortisone/l, 10 mmol glutamine/l, 10 mg insulin/ml, 100 ng hydrocortisone/ml, 10 mg transferrin/ml, 25 ng sodium selenite/ml and 0.1 mg kanamycin/ml.

Binding studies

A full description of the method for measuring EGF receptor binding in human endometrial cells has been given elsewhere (Watson et al., 1994). The cells were washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS) pH 7.5 and then incubated at 37°C for 30 min in Ham’s F10 medium containing 0.1% w/v bovine serum albumin (BSA). The range of concentrations of [125I]-labelled EGF used for Scatchard analysis (Scatchard, 1949) was 0.3–5 nmol/l. The dissociation constant of 5 nmol [125I]-labelled EGF/l. The addition of 1 ml of ice-cold PBS terminated the reaction, and the cells were then washed twice with 0.5 ml of ice-cold PBS. Sodium hydroxide (5 mol/l) (200 µl) was added to solubilize the cells. The supernatant was then transferred to polypropylene tubes and counted in a Hewlett-Packard γ counter. Results were expressed as fmol EGF bound mg/protein. The method of Lowry et al. (1951) was used to measure protein concentrations. Statistical analyses were carried out using analysis of variance (ANOVA) and the unpaired t-test.

Results

The effect of a 6 day treatment with testosterone (0.001–100 µmol/l) on the level of EGF receptor in cultured human endometrial stromal cells is shown in Figure 1. There was a dose related increase in EGF receptor number over the concentration range of 1–100 µmol/l with a maximum response at a concentration of 10 µmol/l. At this concentration, the increase in EGF receptor number was 196 ± 23% control, where the control value was designated to be 100% (P < 0.001), i.e. ~2-fold.

Treatment of cultures prepared from 12 tissue samples with 10 µmol testosterone/l gave a median (range) response of 210% (135–235%) of the control for proliferative phase tissue (n = 7) and 195% (150–215%) of the control for secretory phase tissue (n = 5). There was no significant difference in the response obtained for the two phases of the cycle. In a second experiment, the results of which are shown in Figure 2, cultures were treated for 1–6 days with or without 10 µmol testosterone/l. There was no significant effect of testosterone on EGF receptor synthesis until day 4 when an increase of 188 ± 20% of the control was observed. This was maintained on days 6 and 7 with no further increase (170 ± 22% and 170 ± 12% control respectively).

Scatchard analysis of binding data revealed that testosterone increased the number of EGF receptors without affecting the affinity of the receptor for EGF (Figure 3). The dissociation constant was 1.25 nmol/l in the absence of, and 1.42 nmol/l in the presence of, 10 µmol testosterone/l. The effect of testosterone could be indirect, through its conversion by aromatase to oestradiol, which then increases.
Figure 2. Time course of induction of epidermal growth factor (EGF) receptor by 10 μmol testosterone/l (□) (control, ■). Each value represents the mean ± SD of triplicate determinations. Results are initially expressed as fmol EGF bound/mg protein and presented here as percentage of control where the control value is designated as 100%. This figure is typical of three experiments (**P < 0.001 compared with control).

Figure 3. Scatchard analysis of [125I]-epidermal growth factor (EGF) binding to endometrial stromal cells following 6 days of treatment with 10 μmol testosterone/l (□) (control, ■). This figure is typical of three experiments.

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EGF receptor levels. In order to investigate this possibility, the effect of dihydrotestosterone (DHT), which is not a substrate for aromatase, was examined. The results are shown in Figure 4. DHT caused a dose-dependent increase in the number of EGF receptors over the range 1–100 μmol/l with a maximal effect of 244 ± 6% of the control at a concentration of 10 μmol/l. These results indicate that aromatization of testosterone to oestradiol is not involved in the testosterone-induced stimulation of EGF receptor binding. Moreover, the aromatase inhibitor 4-hydroxyandrostenedione (10 μmol/l) did not affect the testosterone-induced response (Figure 5). However, addition of the anti-androgen hydroxyflutamide (10 μmol/l) to endometrial stromal cell cultures caused a marked inhibition of the testosterone-induced response (Figure 6). The anti-oestrogen ICI182780 (100 nmol/l) had no effect on the testosterone-induced increase in the number of EGF receptors (Figure 7), again indicating that the effect is not mediated by oestrogen.

The relative potencies of seven androgens in stimulating EGF receptor synthesis are shown in Figure 8a. DHT was the most potent androgen tested. In this experiment it caused a stimulation of 205 ± 20% of the control whereas the response to testosterone was 155 ± 35% of the control. Androstenediol, an androgen which also has oestrogenic properties, caused a modest increase of 120 ± 10% control. Dehydroepiandrosterone (DHA), dehydroepiandrosterone sulphate, androstane-diol and androstenedione had no effect.

Figure 8b shows the effect of androgens (10 μmol/l) on glandular epithelial cells. DHT caused an increase of 185 ± 30% of the control and testosterone of 147 ± 5% of the
control where the control was 100 ± 24%. Androstenedione had no effect.

Discussion

The presence of several androgens has been demonstrated in the endometrium. Guerrero et al. (1975) showed that testosterone, androstenedione, androstenediol and DHA are present in the endometrium at higher concentrations than in plasma, but found no tissue/plasma gradient for DHAS. The steroidogenic enzymes aromatase (Tseng, 1984), 17β-hydroxysteroid dehydrogenase (Tseng et al., 1981; Bonney et al., 1985), 5α-reductase and DHA sulphatase (Hausknecht et al., 1982) are present, allowing the interconversion of androgens within the endometrium. The results of the present study suggest that one mechanism by which androgens may be exerting their effects on the human endometrium is by increasing the concentration of the EGF receptor, thus enhancing the action of EGF. Liu et al. (1993) demonstrated that the proliferative effects of testosterone and DHT on human prostate cancer cells are mediated by the EGF receptor. Testosterone, DHT, EGF and transforming growth factor-α (TGF-α) all caused an increase in cell growth, but the effect was greater when the androgens and growth factors were added in combination. Immunoblotting of EGF/TGFα receptor protein, and Northern blot analysis of TGFα and EGF/TGFα receptor messenger ribonucleic acid (mRNA) revealed that the androgens had increased the concentrations of both ligands and receptor.

Several studies have suggested that oestradiol causes an increase in the concentration of the EGF receptor in the human endometrium (Taketani and Mizuno, 1988; Troche et al., 1991; Watson et al., 1996). The activity of aromatase in the endometrium (Tseng, 1984) has led to the suggestion that one mechanism by which androgens may exert their effects is via their conversion to oestradiol which then binds to the oestrogen receptor, thus stimulating endometrial cell growth. Nagamani et al. (1992) postulated this as a means by which the proliferative-
tion of the endometrium of women with endometrial cancer and hyperandrogenism may be stimulated. However, this mecha

ism is unlikely to be of importance in the present study, as the aromatase inhibitor 4-hydroxyandrostenedione did not affect the androgen-induced increase in EGF receptor concentration. Moreover, DHT, which is not a substrate for aromatase, was able to induce an effect. High doses of androgens are also able to exert their effects by causing the translocation of the oestrogen receptor to the nucleus (Rocheft and Garcia, 1976; Schmidt and Katzenellenbogen, 1979). Androstenediol and androstenediol bind to oestrogen receptors with relatively high affinity (Garcia and Rocheft, 1979), while DHT, the most potent androgen, binds more weakly (Zava and McGuire, 1978). The oestrogen receptor is unlikely to be involved in the testosterone-stimulated increase in EGF receptor concentration, however, as the oestrogen antagonist IC1182780 did not diminish the testosterone-induced response. Hydroxyflutamide is a non-steroidal anti-androgen which competitively inhibits the action of testosterone and dihydrotestosterone by binding to androgen receptors. The abolition of the effect of testosterone by hydroxyflutamide confirms that the actions of androgens on the EGF receptor in the endometrium are achieved as a consequence of their binding to the androgen receptor.

The binding of androgen to a specific receptor has been demonstrated in the endometrium by Muechler (1987). The androgen receptor has been localized by immunocytochemistry to the glandular and stromal cells, the functionalis layer showing stronger staining than the basal layer (Horie et al., 1992). Thus androgens have the potential to exert direct effects on the endometrium via binding to their own receptor. The concentration of the endometrial androgen receptor does not appear to vary according to the stage of the menstrual cycle (Horie et al., 1992). In the present study, the increase in EGF receptor concentration stimulated by the same dose of testosterone was similar in tissue taken from women in the proliferative and in the secretory phases. Taketani and Mizuno (1988) and Troche et al. (1991) showed that the concentration of EGF receptor in endometrial homogenates was raised around the middle of the menstrual cycle. This may be due, in part, to the mid-cycle increase in serum testosterone concentrations (Abraham, 1974). A study by Iwai et al. (1995) demonstrated that testosterone reduced the expression of androgen receptor mRNA in cultures of endometrial stromal cells. Moreover, testosterone was able to reduce the level of both oestrogen and progesterone receptor mRNA in these cells. This may add further complexity to the regulation of EGF receptor concentrations by the ovarian steroids, since EGF receptor synthesis is also stimulated by oestrogen and progesterone (Taketani and Mizuno, 1991; Watson et al., 1996).

The question remains whether the increased concentration of serum androgens in women with PCO has an adverse effect on the development of their endometrium. Clearly, the endometrium of women with PCO who are ovulating is thicker than that of women with normal ovaries (Adams et al., 1988). Furthermore, the incidence of endometrial cancer is increased in women with conditions such as PCO which are characterized by hyperandrogenism, suggesting that androgens are involved in the regulation of endometrial growth. Nagamani et al. (1992) showed that the production of androstenedione, testosterone and dehydroepiandrosterone by ovarian stroma was increased in postmenopausal women with endometrial cancer compared to women without cancer. The uterotrophic effects of androgens have been demonstrated in vivo in immature female rats (Schmidt and Katzenellenbogen, 1979). However, these findings are contradicted by evidence that androgens inhibit human endometrial proliferation in vitro (Neulen et al., 1987; Rose et al., 1988), and the therapeutic suppression of endometriosis by the administration of androgenic drugs such as danazol.

Androgens have now been implicated in the control of endometrial differentiation, and in embryo implantation. In human endometrial stromal cell cultures, testosterone and DHT stimulated the production of prolactin, a biochemical marker used to evaluate endometrial differentiation (Narukawa et al., 1994). Histological changes in the uteri of ovariectomized rats were noted following the administration of testosterone. These included an increase in glycogen content, total protein and RNA (Datta et al., 1974). Chandrasekhar et al. (1990) found that the anti-androgen hydroxyflutamide was able to delay implantation, fetal development and parturition in pregnant rats, and to suppress decidualization in pseudopregnant rats. Furthermore, hydroxyflutamide inhibits preimplantation mouse embryo development, an effect which can be reversed by testosterone, but not by oestrogen or progesterone (Yallampalli et al., 1993). The importance of EGF in the process of implantation is indicated in a study by Johnson and Chatterjee (1993) in which EGF induced embryo implantation in the rat. This is substantiated by the presence of increased EGF receptors at the preimplantation sites of mouse uteri (Brown et al., 1989). An increase in pre-pro EGF mRNA has also been observed in the decidua of human pregnancy (Sakakibara et al., 1994). The present study provides a link between the influence of EGF and androgens on implantation by suggesting that the effect of testosterone may be mediated by the EGF receptor.

Several growth factors and their receptors have been shown to be involved in the regulation of endometrial growth and differentiation. Platelet-derived growth factor (PDGF) is mitotic to cultured endometrial stromal cells, moreover, EGF and PDGF enhance each others’ mitogenic action (Chegini et al., 1992). The insulin-like growth factor-I (IGF-I) gene is expressed primarily in proliferative and early secretory endometrium, indicating that IGF-I mediates oestrogen action. The insulin-like growth factor-II (IGF-II) gene is expressed in mid to late secretory endometrium, suggesting that IGF-II may be involved in endometrial differentiation (Giudice et al., 1993). Fibroblast growth factor (FGF) has been identified in endometrial glandular epithelium (Cordon-Cardo et al., 1990) and is a potent mitogen for cultured endometrial stromal cells (Irwin et al., 1991). The expression of transforming growth factor-β (TGF-β) mRNA is lowest during the proliferative phase, increases two-fold in the secretory phase and five-fold in early pregnancy decidua (Kauma et al., 1990) suggesting that it may contribute to the inhibition of cellular proliferation during periods in which cellular differentiation is dominant.


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