Effects of androstenedione, insulin and luteinizing hormone on steroidogenesis in human granulosa luteal cells

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BACKGROUND: The present study was conducted to investigate the effect of androstenedione, insulin and LH on human granulosa cell oestrogen and progesterone production. We postulated that elevated concentrations of androstenedione, insulin and LH may be important modulators of granulosa cell steroidogenesis. METHODS: Granulosa cells obtained in connection with IVF procedures were cultured for a total of 4 days in serum-free medium containing androstenedione (10⁻⁶ mol/l). We tested the effect of androstenedione (10⁻⁵ mol/l) on insulin (0–800 µIU/ml), LH (1–10 ng/ml) as well as on insulin /c⁹⁰⁵⁹/LH-stimulated oestrogen and progesterone production.

RESULTS: Insulin increased the basal secretion of steroid hormones, and furthermore augmented LH-stimulated oestrogen and progesterone accumulation in granulosa cell cultures. Androstenedione (10⁻⁵ mol/l) stimulated basal oestrogen production, but significantly reduced (32–58%) insulin /c⁹⁰⁵⁹/LH-stimulated oestrogen and progesterone secretion (P < 0.05). CONCLUSION: These results suggest that high androstenedione concentrations may act directly to impair insulin augmentation of LH-stimulated oestradiol and progesterone production in cultured human granulosa luteal cells. This is compatible with the hypothesis that high androgen levels may inhibit oestrogen production in polycystic ovary follicles, and as such may contribute to anovulation and infertility in women with polycystic ovary syndrome.

Key words: androgen/granulosa cell/insulin/polycystic ovary syndrome (PCOS)/steroidogenesis

Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine disorder characterized by hyperandrogenaemia and anovulatory infertility. It is associated with hyperinsulinaemia, insulin resistance and hypersecretion of LH (Dunaif et al., 1989; Franks, 1995; Dunaif, 1997). Although it is the most common cause of anovulatory infertility (Franks, 1995), the mechanism remains unknown.

It has been suggested that the granulosa cells of the PCO follicles may be abnormal (Erickson et al., 1992), but following their culture in serum-free medium in vitro, they were capable of producing normal or increased amounts of oestradiol in response to FSH stimulation (Erickson et al., 1992; Mason et al., 1994). However, although the PCO follicles contain increased amounts of FSH (Erickson et al., 1992), several studies have found the oestrogen concentration in these follicles to be normal or low compared with that found in normal dominant follicles (Erickson et al., 1992; San Roman and Magoffin, 1992; Mason et al., 1994). These observations suggest that substances in the microenvironment of the PCO follicles may modulate granulosa cell steroidogenesis.

Excessive androgen production is a universal finding in PCO ovaries. PCO ovaries produce significantly more androstenedione than theca cells from normal follicles (Gilling-Smith et al., 1994); moreover, higher androstenedione concentrations have been found in PCO follicles than in normal follicles (Mason et al., 1994; Franks et al., 1998). Although androstenedione is the most important substrate for oestrogen production, a high androgen concentration in the PCO follicle is also believed to be involved in the pathogenesis of follicular development arrest (Hillier, 1987; Hillier and Tetsuka, 1997). Hyperinsulinemia and insulin resistance are important PCOS features (San Roman and Magoffin, 1992; Dunaif, 1997), and at the cellular level, insulin has specific actions on steroidogenesis which are effected through its own receptor (Willis and Franks, 1995). These actions seem to be preserved in insulin-resistant states, and the ovary does not appear to be insulin resistant in women with PCOS (Willis et al., 1996; Willis and Franks, 1995).

Hypersecretion of LH is also characteristic in women with PCOS (Franks, 1989), due to increased frequency and amplitude of LH release (Rebar et al., 1976; Waldstreicher et al., 1988). Although LH is essential for oestrogen synthesis and maintenance of follicular growth, excessive stimulation may adversely affect normal follicular development (Chappel and Howles, 1991; Overes et al., 1992).
Hormonal imbalance and elevated concentrations of androstenedione, insulin and LH may thus be important factors modulating granulosa cell steroidogenesis and affecting follicle maturation in PCOS.

The purpose of the present study was therefore to investigate the granulosa cell steroidogenesis, with particular reference to the interactions between high concentrations of androstenedione and elevated concentrations of insulin and LH.

Material and methods

Reagents and hormones
Human LH and 4-androstene-3,17-dione were purchased from Sigma Chemical Co. (St Louis, MO, USA). Novo Nordisk (Bagsværd, Denmark) supplied human insulin. Ficoll-Paque was supplied by Pharmacia & Upjohn (Stockholm, Sweden). Medium 199 was obtained from Life Technologies, (Paisley, UK). Oestradiol-17β and progesterone radioimmunoassay kits were obtained from Wallac Oy, (Turku, Finland). Human plasma albumin was purchased from Statens Serum Institut (Copenhagen, Denmark). Flushing medium was obtained from Medicol (Jyllinge, Denmark).

Cell culture

Human granulosa cells were obtained from follicular aspirates of women undergoing IVF due to tubal pathology. Individuals with PCOS were excluded following clinical examination. The patients were treated with gonadotrophin-releasing hormone (GnRH) agonist for 16 days before inducing follicular development with recombinant FSH. Follicle aspiration was performed 37 h after administration of human chorionic gonadotrophin (HCG).

After removal of the oocyte–cumulus complex, the granulosa cells were retrieved from the follicular fluid and subsequently layered on a Ficoll-Paque gradient and centrifuged at 1000 g for 20 min to pellet the red blood cells. Aliquots were taken for cell counting, and the granulosa cells were plated at a density of 20 000/well on 96-well culture plates. The cells were cultured in medium 199 with Earl’s salts and 1-glutaminate, 27 mmol/l bicarbonate, 50 mg neomycin/l and 2 μg fungilin/l supplemented with 0.1% human albumin and 10–6 mol/l androstenedione. The granulosa cells were incubated in a final volume of 200 μl at 37°C in a humidified atmosphere containing 5% CO2 balance air. Cells were preincubated for 2 days, after which the medium was changed, then cultured for 2 days with different combinations of androstenedione (10–6–10–5 mol/l), insulin (0–800 μIU/ml) and LH (1–10 ng/ml). The amount of oestradiol in the medium was measured using radioimmunoassay in which the sensitivity was 0.05 nmol/l, and the intra-assay and interassay coefficients of variation were 4.2 and 3.6% respectively. When the same technique was used to measure progesterone, the kit showed a sensitivity of 0.8 nmol/l. The intra-assay and interassay coefficients of variation were 1.8 and 1.9% respectively. Granulosa cell proliferation was evaluated by [3H] thymidine labelling technique as previously described (Ovesen et al., 1994).

Cells from 14 patients were used, and each experiment was based on cultures of granulosa cells derived from all preovulatory follicles >15 mm from one patient. The experiment was performed at least three times with granulosa cells from three different women, and there were three observations in each experiment.

Androstenedione

Androstenedione was added to all cultures as a substrate. In cultures where a high androgen concentration was intended, 10–5 mol/l androstenedione was used; in all other cultures 10–6 mol/l androstenedione was added.

Statistics

The results are expressed as means ± SEM for triplicate culture wells from three or more experiments. Statistical differences between groups were analysed by ANOVA. Specific differences in steroid production between treatments were determined using Student’s t-test. Statistical significance was determined as P < 0.05.

Results

Oestrogen production

Insulin stimulated oestradiol production in human granulosa luteal cells using a serum-free system containing 10–6 mol/l androstenedione (P < 0.001) (Table I). Cells incubated with 10 ng/ml LH secreted significantly more oestradiol than controls (P < 0.01), whereas LH 1 ng/ml showed no stimulatory effect (Figure 1).

When both LH (1 ng/ml) and insulin (200, 400, 800 μIU/ml) were added to the media, insulin increased LH-stimulated oestrogen accumulation in an additive manner (Figure 1) (only data for LH 1 ng/ml + insulin 400 μIU/ml is shown). Moreover, insulin (200, 400, 800 μIU/ml) combined with LH

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Figure 1. Additive stimulatory effect of LH and insulin on oestrogen production. The black columns show the effect of LH alone. Grey columns illustrate the stimulatory effect of 400 μIU/ml insulin alone and in combination with LH. Each column represents the mean ± SEM of three independent experiments with three replicates per experiment.
Effect of androstenedione, insulin and LH on steroidogenesis

Figure 2. Effect on the oestrogen production of different combinations of insulin, LH and androstenedione (Adione). The black columns illustrate the stimulatory effect of insulin (0–800 µIU/ml). The light-grey columns show the effect of 10 ng/ml LH alone and in combination with rising concentrations of insulin, and the dark-grey columns represent the inhibitory consequence of androstenedione (10⁻⁵ mol/l) on insulin/LH stimulated estrogen production in human granulosa cells. Each column represents the mean ± SEM of three independent experiments with three replicates per experiment.

Figure 3. Effect of androstenedione (Adione) (10⁻⁶ and 10⁻⁵ mol/l) on basal and stimulated (200 µIU/ml insulin and 10 ng/ml LH) estrogen production in human granulosa cells. Each column represents the mean ± SEM of three independent experiments with three replicates per experiment.

Of special importance was the observation that high concentrations of androstenedione (10⁻⁵ mol/l) stimulated oestradiol production in a synergistic manner (Figure 2).

Of special importance was the observation that high concentration of androstenedione (10⁻⁵ mol/l) significantly reduced the LH + insulin stimulated oestradiol production (32–58%) compared with cultures containing 10⁻⁶ mol/l of androstenedione (P < 0.05). Nevertheless, 10⁻⁵ mol/l androstenedione elevated the basal oestradiol concentration (P < 0.01) (Figure 2).

When LH or insulin was added separately, the level of oestradiol was not affected by changes in androstenedione concentration (Figure 3). The proliferation experiments showed that the effect of insulin, LH and androstenedione on steroid production were independent of any possible mitotic properties of the hormones. There were no significant granulosa cell proliferation using a serum free medium (data not shown).

Progesterone production

Progesterone production was stimulated by the higher concentrations of insulin (400 and 800 µIU/ml) (P < 0.05) (Figure 4). Cells incubated with LH (10 ng/ml) secreted significantly more progesterone compared with controls (P < 0.01) (Figure 4). Furthermore, insulin (400 and 800 µIU/ml) increased LH-stimulated (10 ng/ml) progesterone accumulation in an additive manner (P < 0.05). Interestingly this additive stimulation was significantly inhibited by 10⁻⁵ mol/l androstenedione (10–47%) (P < 0.05) (Figure 4), even though cultures containing androstenedione 10⁻⁵ mol/l secreted significantly more progesterone than cultures containing 10⁻⁶ mol/l (P < 0.01) (data not shown). Progesterone production following stimulation by LH (10 ng/ml) or insulin (400 and 800 µIU/ml) separately was not influenced by elevated androstenedione concentration. The lower concentrations of LH and insulin did not affect progesterone secretion (data not shown).

Discussion

The present study shows that insulin not only causes increased steroidogenesis in human granulosa luteal cell cultures, but also augments LH-stimulated oestradiol and progesterone production in a synergistic manner. The data that demonstrate that this synergism can be inhibited by androgen at concentrations similar to those found in PCO follicles are of particular interest.

Previous studies have demonstrated that granulosa cells from both normal and PCO follicles are responsive to insulin, which acts via its own receptors (Willis and Franks, 1995; Willis et al., 1996), suggesting that the ovary is not insulin...
resistant in women with PCOS. Insulin has been demonstrated to enhance the induction of LH receptors in granulosa cells from rats (Adashi et al., 1985). Moreover, Willis et al. found insulin to enhance LH-induced oestrogen and progesterone production in an additive manner in human granulosa cells from normal and PCO follicles (Willis et al., 1996).

As a consequence, PCO follicular fluid may be expected to contain increased amounts of oestrogen to such an extent that extrapolation from the above mentioned studies (Willis and Franks, 1995; Willis et al., 1996) is appropriate. However, the fluid from PCO follicles contains less oestrogen than size-matched normal follicles (San Roman and Magoffin, 1992; Agarwal et al., 1996). This difference is not caused by lack of FSH, since studies have shown that PCO follicles contains higher amounts of this hormone than controls, and in addition in-vitro studies show that the granulosa cells are capable of releasing normal or increased amounts of oestradiol in response to stimulation by FSH (Erickson et al., 1992). Consequently, inhibition of the synergism between insulin + LH seems to be present in the follicle microenvironment and may well be ascribed to the elevated androgen concentration found in the PCO follicles.

Excessive androgen production is a universal finding in PCOS, and androgen levels are also elevated in fluid from PCO follicles. Studies have shown that the range of androstenedione in follicular fluid of PCOS is 1592-3033 ng/ml (5.56–10.59 mol/l) (Mason et al., 1994; Agarwal et al., 1996). Consequently, in the present study 10⁻⁶ mol/l of androstenedione was added to cultures in order to imitate PCOS conditions and the results were compared with cultures containing 10⁻⁶ mol/l androstenedione to mimic the normal situation. We demonstrate that high androstenedione level inhibits the additive effect of insulin and LH significantly. A relatively small increase of androgen levels seems to switch the hormone from a stimulatory to an inhibitory action. Consequently, concentrations of androgen similar to that found in fluid of PCO follicles significantly impair insulin + LH stimulatory effects.

These results are new, and even though precaution has to be taken when extrapolating from data obtained in cultures of granulosa luteal cells to the preovulatory follicle function in PCOS, the present observations add new information on the hormonal interaction between androstenedione, insulin and LH.

Others have also investigated the effect of androgen on oestrogen synthesis in human granulosa cell. Agarwal et al. demonstrated that 5α-reduced metabolites of androstenedione are found in significantly higher concentrations in PCO follicular fluid than in controls, and that the substance is able to inhibit oestrogen production in granulosa cells, probably as a competitive inhibitor of the aromatase enzyme (Agarwal et al., 1996). However, in our set-up the decline in steroid secretion, in response to high androstenedione concentration is more likely to represent substrate inhibition of the aromatase activity.

Even though LH is essential for oestrogen synthesis and maintenance of follicular growth, excessive amounts may adversely affect normal follicular development (Chappel and Howles, 1991; Overes et al., 1992). It has been suggested that follicles exposed to inappropriately high concentrations of LH initiate a premature luteinization which may compromise oocyte development (Hillier, 1994). This effect is thought to be achieved through activation of intracellular cAMP (Franks et al., 1998), but it is not known whether insulin amplifies this action of LH. However, androgens have been shown to augment gonadotrophin-induced cAMP production in granulosa cell cultures (Harlow et al., 1988), indicating that high androgen level may contribute to premature maturation of the follicle.

Both insulin and LH are known to stimulate androgen production from theca cells (Nestler and Strauss, 1991; Gilling-Smith et al., 1994). This effect of LH + insulin may lead to a high intra follicular concentration of androgen, which may impair the stimulatory effect of LH + insulin on granulosa cell oestrogen production.

Of course, these in-vitro results cannot be transferred directly to the clinical and pathological situation in PCOS. Nevertheless our data are compatible with the concept that increased levels of androgen may compromise development of follicles and contribute to premature maturation of the oocyte in women with PCOS.

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References


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