Metformin has direct effects on human ovarian steroidogenesis

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Objective: To investigate the possibility of direct effects of metformin on ovarian steroidogenesis.

Design: Cultured ovarian cells.

Setting: Academic research environment.

Patient(s): Women undergoing bilateral salpingoophorectomy for benign gynecological disease.

Main Outcome Measure(s): Estradiol and P were measured in granulosa cell (GC) conditioned medium and androstenedione (A) and P in theca conditioned medium.

Result(s): The effect of addition of metformin alone to GCs was variable, but significant inhibition of both P and E2 was seen (range 0%–30%). Metformin dose-dependently inhibited gonadotrophin and insulin-stimulated P and E2 production (range 25%–50%). In theca, metformin inhibited A production (0%–40%) with no effect on P. In the presence of insulin, A was inhibited dose-dependently and P increased by a similar magnitude.

Conclusion(s): These results demonstrate a direct effect of metformin on ovarian steroidogenesis. The inhibitory effects on androgen production in particular would be beneficial in polycystic ovary syndrome (PCOS). (Fertil Steril 2003;79:956–62. ©2003 by American Society for Reproductive Medicine.)

Key Words: Metformin, polycystic ovary syndrome, steroidogenesis

Polycystic ovary syndrome (PCOS) is the commonest endocrine disorder in women of reproductive age (1). It is characterized by hyperandrogenism, often in conjunction with obesity and oligomenorrhea (2). The ovaries are bilaterally enlarged and contain numerous subcapsular follicles (3), the theca of which constitutively secretes increased amounts of androgen (4, 5). Women with this condition are often insulin resistant (6, 7) and there is a clear link between the degree of insulin resistance and anovulation (8). The compensatory hyperinsulinemia further stimulates ovarian androgen production (9) and may also be detrimental to granulosa cell function (10, 11).

Until recently treatment of PCOS was largely symptomatic, but there has been a move toward correction of the underlying insulin resistance in those women for whom this is a confounding factor. Initially these treatments were by diet alone (12), but more recently insulin sensitizers have been studied such as troglitazone and, following the latter’s withdrawal, metformin. These studies have reported varying results (13, 14), but a significant number demonstrated a return to regular cycles and a reduction in insulin levels and circulating androgens (15–24) and its use has also proven beneficial in clomiphene-resistant women (25) and in adolescents with hyperandrogenism (26), (see 27 for review). This lead us to postulate that metformin was exerting direct effects on ovarian steroidogenesis. The aim of this study was to investigate this possibility.

MATERIALS AND METHODS

Patients
Ovaries were obtained from women undergoing oophorectomy for nonovarian gynecological disease. All except one patient with ovulatory polycystic ovary (PCO) reported regular cycles; cycle stage was random and none had received medication for stimulation or suppression of ovarian function for at least 3 months before surgery. Approval for this study...
was granted by the local ethics committees and by the Ethics Committee of The Faculty of Medicine and Surgery, Medical School, Malta. Informed consent was obtained from each patient before surgery. Morphology was assigned according to previously published criteria (28). A series of experiments was also carried out using luteinized granulosa cells (GC) collected at the time of oocyte aspiration from patients undergoing ovarian stimulation for in vitro fertilization.

**Cell Cultures**

Follicles were dissected intact from the ovaries microscopically, the diameter measured, and follicular fluid aspirated. Experiments were carried out on pooled cells from a single patient or on cells from individual follicles where stated. Details of the size of follicles dissected from each pair of ovaries and the sizes from which cells were pooled for each experiment are shown in Table 1.

**Granulosa Cells**

Granulosa cells were harvested and cultured as previously described (28). Approximately $5 \times 10^4$ viable cells/well were incubated in a 200-$\mu$L volume of serum-free Medium 199 (Invitrogen, Paisley, Scotland) with the addition of antibiotics (penicillin and streptomycin, Invitrogen) and 200 mM L-glutamine. Incubations were carried out in the presence of $10^{-7}$ M T (Sigma Chemical Co., Poole, England) as an aromatase substrate. The luteinized GCs were separated from blood cells and other debris using the method of Abeyesekara et al. (29), plated with 5% fetal calf serum (FCS) for 48 hours, and after removal of this medium, cultured in the same manner as unluteinized cells, but without the addition of T.

**Theca Cells**

The theca “shells” remaining after the removal of GCs from incised follicles were scraped carefully to remove any adhering GCs. The theca was then peeled from the remaining stroma, pooled from individual follicles, finely chopped and subjected to enzymatic dispersion into single cells using a modification of the method of Campbell et al. (30). Briefly, an enzyme solution containing 50 mg of collagenase, 10 mg each of hyaluronidase and protease, 200 $\mu$L of a 2-g/L solution of DNase (all enzymes from Sigma) in 10 mL of Dulbecco’s phosphate-buffered saline (PBS) without calcium or magnesium (Invitrogen) was filter sterilized. Finely minced theca was added and the mixture rotated at 37°C until dispersion was achieved at which point 1 mL of FCS was added to inhibit the reaction. Final dispersion was accomplished by flushing the cell mixture with a syringe. Viable cells ($5 \times 10^5$) were incubated in 200 $\mu$L of serum-free Medium 199 with additions as above.

**Experimental Protocol**

Incubations were carried out in the presence or absence of a range of doses ($10^{-12}$–$10^{-6}$ M) of metformin (Sigma), diluted to the requisite concentration in medium, with or without purified pituitary FSH (5 ng/mL) (Endocrine Services, Bideford-on-Avon, UK) or insulin (10 ng/mL), the latter to mimic possible conditions in women with hyperinsulinemia. Each dose was added to between three and six wells in each experiment and all treatments were added simultaneously. Cultures were performed for 48 hours. Steroids in the medium were measured by RIA as previously described (4, 28). Results are expressed as nanomoles per 1,000 cells at the start of the experiment and in two cases the relative number of cells at the termination of the experiment was determined by mitochondrial tetrazolium salt (MTT) assay (Celltiter 96, Promega, Southampton, UK).

**Analyses**

To combine the results of multiple experiments in GCs for analysis, the percentage change from basal produced by each treatment was calculated with basal (control) levels being taken as 100%. A similar calculation was made for Table 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Age (y)</th>
<th>Ovarian morphology</th>
<th>Menstrual cycle (day/length)</th>
<th>No. follicles dissected (size range)</th>
<th>Follicle sizes pooled in experiment [no. (size range)]</th>
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<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>ovPCO</td>
<td>8/28</td>
<td>16 (3–14)</td>
<td>1 (14)</td>
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<tr>
<td>1</td>
<td>43</td>
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<td>8/28</td>
<td>9 (3–20)</td>
<td>1 (20)</td>
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<td>1</td>
<td>30</td>
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<td>10/28</td>
<td>6 (3–8)</td>
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<tr>
<td>3a, b</td>
<td>48</td>
<td>ovPCO</td>
<td>30/32</td>
<td>17 (2–9)</td>
<td>6 (6–9)</td>
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<td>30</td>
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<td>10/28</td>
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*ovPCO = ovulatory PCO; *Irreg = irregular.

results in luteinized GCs. Results were compared by analysis of variance (ANOVA) with Bonferroni’s post-hoc test.

For theca experiments, the effects of metformin on steroid production were analyzed by ANOVA as above for the dose response. To combine results at single doses in three separate experiments, percentage change was calculated as for the GC experiments and the mean and standard error plotted. These results were compared using Student’s t-test.

RESULTS

Although we have insufficient data in the two groups to draw any firm conclusions, there were no obvious differences in responses between patients with normal or PCO for either granulosa or theca and data were therefore pooled for statistical analysis.

Granulosa Cells

Levels of steroid in the medium ranged from 3 to 85 and from 2.6 to 95 nmol/1,000 cells/48 hours for E₂ and P, respectively, across the three experiments. Metformin added in the presence of T alone was inhibitory of basal P production, but had no consistent effect on basal E₂ in three experiments in unlueteinized GCs (Fig. 1). Follicle-stimulating hormone stimulated the production of both steroids in all three experiments. Metformin consistently inhibited FSH-stimulated steroid production by these cells (estradiol \( P < .002 \), progesterone \( P < .0001 \), ANOVA), with P being suppressed to levels below those seen with metformin alone. There was no significant effect on cell number at the termination of the experiment as judged by MTT assay.

Insulin significantly stimulated E₂ production and this was inhibited in a dose-dependent manner by metformin (\( P = .05 \)). There was no stimulation of P production by insulin in the same cells, but P production was inhibited in the presence of insulin and metformin (\( P < .001 \)). In luteinized GCs there was a clear, dose-dependent inhibition of both E₂ and P production by metformin (Fig. 2).

Theca Cells

A dose response to metformin in the presence or absence of insulin was performed in cells from pooled follicles. Although there was some degree of variability, metformin inhibited basal androstenedione (A) production at a dose of \( 10^{-7} \) M (Fig. 3A). There was a significant increase in A in response to insulin in these cells, and metformin significantly inhibited A production in a dose-dependent manner in the presence of insulin. In contrast, metformin had no effect on basal P levels, but increased P accumulation in the medium in a dose-dependent manner in the presence of insulin (Fig. 3B). The levels of P secreted were approximately ten-fold higher than A.

Three other experiments were carried out in theca cells using a single dose of metformin, \( 10^{-8} \) M and in two, sufficient cells were obtained to investigate the effect of this dose in the presence of insulin (10 ng/mL). These results were combined with those from Figure 3 and the mean and SE of the results as a percentage of control values is shown in Figure 4.
Metformin significantly inhibited A and P production (P < .04 and < .02, respectively, Student’s t-test) in theca cultures. Insulin stimulated the production of both steroids but, whereas metformin inhibited the insulin-induced stimulation of A back to basal levels, P production was enhanced to approximately twice that seen in the presence of insulin alone; however, this was not significant due to the wide SE.

In each case, in theca cells cultured in the presence of insulin, the magnitude of the effect of metformin on P was approximately equal and opposite to that seen on A.
DISCUSSION

In this study we have demonstrated a direct effect of metformin on steroidogenesis by human ovarian cells in culture. The effects were seen on both GC and theca cells and were particularly clear in cells stimulated by insulin and in cells which were from preovulatory follicles and therefore luteinized.

The inhibitory effects on P in GCs suggest that metformin has an effect on, or upstream of, 3βHSD in the steroid enzyme pathway. Before its withdrawal due to hepatotoxicity, the thiazolidinedione troglitazone had also been shown to be effective both in increasing insulin sensitivity in women with PCOS and in reducing elevated androgen levels (31). Although the mechanism of action of troglitazone as an insulin sensitizer is unclear, it has also been shown to inhibit P production by porcine GCs in culture (32). That the effect was specifically on 3βHSD was evidenced by the fact that pregnenolone levels increased in the same wells. Troglitazone had no effect on aromatase in porcine cells.

It has also been reported that thiazolidinediones inhibited P450c17 and 3βHSD activity in a “humanized” yeast expression system, but that metformin had no effect (33). This system uses yeast cells carrying the enzymes of interest contained in native microsomes. The reason for the difference between our results is not clear but is presumably due either to the fact that the mechanism of action is different in intact ovarian cells or is a consequence of the culture conditions in our experiments. The possibility also exists that the effects we are seeing are not directly on the enzymes in question as we have only measured the steroid products; however, this seems unlikely. In contrast, a second publication directly supports our data in that it demonstrated the inhibition of A and T production by a human ovarian theca-like tumor cell line (34). In these studies there was an inhibition of steroidogenic acute regulatory protein and 17β-hydroxylase in cells stimulated with forskolin; however, the doses of metformin required to achieve this activity were in the micromolar range.

Interestingly, although metformin was consistently inhibitory of insulin-stimulated E2 and P production in our GC experiments, it had approximately equal and opposite effects on P and A production by theca. That the magnitude of these changes was so similar indicates that in the insulin-stimulated theca cells, the inhibition of A production resulted in accumulation of the substrate P. This accumulation presumably overides any inhibitory effect on 3βHSD or enzymes further back in the pathway. It is reasonable to assume that the effect of metformin in these cultures was on P450c17.

In our GC cultures, inhibition of both E2 and P was presumably seen because P is the secreted end point in the steroid synthesis pathway in these cells lacking P450c17 and androgen substrate for aromatase is either being provided in the culture medium, in the case of cells from small follicles, or is converted from androgen accumulated in vivo in the case of luteinized cells. That inhibition of aromatase was also seen indicates that metformin may have actions at multiple sites in the steroid hormone synthesis pathway. Clearly, it will be interesting in the future to investigate the precise sites of action in more detail.

Although the effects of metformin treatment of women with PCOS are variable, the majority report reduced levels of
androgens. In the same studies insulin sensitivity improved and therefore, circulating basal and stimulated insulin levels were also lowered. It has generally been supposed that the main effects of metformin on androgens was by reducing the amount of insulin available to stimulate theca production. Although this must clearly be one mechanism by which metformin is acting in these patients, we have here demonstrated a direct effect of this drug on ovarian hormone synthesis. It has often been those patients with the most marked hyperandrogenism that have benefited most from metformin treatment (19, 20, 24) and interestingly, this group has not always been the most hyperinsulinemic.

That metformin has a direct effect on P450c17 in theca is further evidenced by in vivo studies of stimulated ovarian steroid production before and after metformin treatment. Stimulation of ovarian function with hCG demonstrated reduced 17-α hydroxyprogesterone (17-OHP) in patients following metformin therapy, when compared to baseline testing (22, 35). The responses to ACTH to 17-OHP, T, and A were also lower after metformin treatment (36), more especially, both the ratio of 17-OHP to P which indicates 17-α hydroxylase activity and the ratio of A to 17-OHP, which indicates 17, 20 lyase activity were significantly lower. We have unfortunately been unable to measure steroid intermediates in the theca cell cultures as insufficient volumes of conditioned medium remained.

P450c17 has been shown to be one of the constitutively overactive enzymes in PCOS in a number of studies (5, 37, 38). In the study by Gilling-Smith et al. (4), theca from both ovulatory and anovulatory ovaries was shown to secrete considerably higher amounts of androgen than that from normal ovaries. However, we found suppressive effects on theca from both normal and polycystic ovaries. Our study was not designed to investigate differential effects of metformin on cells from normal low-androgen secreting theca or high-androgen secreting theca from polycystic ovaries. The cells from ovulatory PCO have been included because they are a suitable model in which to test the metformin effects and because they did not show any significant differences in response from cells from normal ovaries.

Although the levels of metformin that follicular cells are exposed to in women on oral metformin treatment are unknown, we surprisingly saw effects in the in vitro cultures with doses as low as 10⁻¹² M and consistently with 10⁻⁸ M, which might be expected to be at or below the physiological range.

In summary, we have shown that metformin has a direct effect on steroidogenesis by ovarian cells in culture. The inhibitory effects on androgen production would be beneficial in PCOS and may contribute to the androgen-lowering effects of this drug in these patients. The precise sites of action of metformin on steroidogenic enzymes and the mechanism of its effects remains to be determined.

References


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