CLINICAL STUDY

The effects of long-term metformin treatment on adrenal and ovarian steroidogenesis in women with polycystic ovary syndrome

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Abstract

Objective: To evaluate adrenal and ovarian steroidogenesis before and after long-term treatment with metformin in women with polycystic ovary syndrome (PCOS).

Design and Methods: Twenty-four women with PCOS were evaluated before and after treatment (27 ± 4 weeks) with metformin (1000 mg/day) using adrenocorticotrophin (ACTH), GnRH analogue and oral glucose tolerance (oGTT) tests. For statistical evaluation, ANOVA and Wilcoxon's test were used.

Results: In 58% of the women a significant improvement in menstrual cyclicity was observed. No significant change in basal steroid levels was found. After ACTH stimulation, a significant decrease in the activity of 3β -hydroxysteroid dehydrogenase in C_{21} steroids ($P < 0.05$) and in 17β -hydroxysteroid dehydrogenase ($P < 0.01$) was observed, as was an increase in the activity of C17,20-lyase in the Δ^4 pathway ($P < 0.01$). A significant growth in the dehydroepiandrosterone (DHEA)/DHEA-sulfate ratio ($P < 0.05$) was detected. With regard to ovarian steroidogenesis, a significant decrease in the stimulated levels of testosterone ($P < 0.05$), index of free testosterone ($P < 0.01$), LH ($P < 0.05$) and oestradiol ($P < 0.01$), and an increase in the levels of 17-hydroxypregnenolone ($P < 0.05$) were detected. In the indices of ovarian enzyme activities, we observed a significant decrease in 3β -hydroxysteroid dehydrogenase in C_{21} steroids ($P < 0.01$), in C17,20-lyase in the Δ^5 pathway ($P < 0.01$), in 17β -hydroxysteroid dehydrogenase ($P < 0.05$) and in aromatase. In glucose metabolism, a tendency towards reduction in the homeostasis model assessment (HOMA)-R (for insulin resistance) and HOMA-F (for β cell function) was detected. In addition, an increase in the levels of C peptide during oGTT was observed ($P < 0.01$).

Conclusions: Long-term metformin treatment reduced various steroid enzymatic activities both in the ovary and the adrenal glands, without apparent changes in basal steroid levels and in insulin sensitivity.

European Journal of Endocrinology 144 619–628

Introduction

Polycystic ovary syndrome (PCOS) is probably the most common endocrinopathy in women of childbearing age. It is invariably associated with hyperandrogenism and, in many cases, with insulin resistance. Insulin has a direct stimulatory effect on ovarian steroidogenesis *in vitro* (1), decreases the synthesis of sex hormone-binding globulin (SHBG) in the liver (2), and inhibits liver production of insulin-like growth factor-binding protein-1, resulting in an increased bioavailability of insulin-like growth factor-I (2). PCOS is commonly considered in relation to the increased ovarian activity of C17-hydroxylase, and to the relatively less stimulated activity of C17,20-lyase (3); insulin is probably involved

in their regulation. Both of these enzyme activities are also important for adrenal steroidogenesis. It is possible that insulin (4) or oestrogens (4–6) are involved in the regulation of these enzymes in the adrenal glands. The origin of hyperandrogenism in women with PCOS has not yet been clearly discerned. A large number of women with PCOS, perhaps as many as 40–60% (3, 7) have, in addition to ovarian hyperandrogenism, signs of adrenal androgen excess, defined for example by high levels of dehydroepiandrosterone-sulfate (DHEAS).

Metformin as an insulin-reducing agent is considered to be a promising treatment for women with PCOS. It has multiple mechanisms of action, including the inhibition of gluconeogenesis in the liver, and the stimulation of peripheral glucose uptake (8); interestingly, there is also

some evidence for the direct inhibitory effect of metformin on ovarian steroidogenesis (9). The literature to date contains only few data about the effects of metformin on adrenal steroidogenesis in women with PCOS (10, 11).

The aim of this study was to evaluate the effect of metformin on both ovarian and adrenal steroidogenesis in long-term therapy in women with PCOS. The majority of studies with insulin-sensitizing agents have used basal steroid levels before and after such therapy with contradictory results, either finding a reduction in androgens (12–18) or not finding such a reduction (19–21). We attempted the use of the product/precursor ratios of various steroids after specific stimulation (adrenocorticotrophin (ACTH) test for adrenals and gonadotrophin-releasing hormone (GnRH) analogue (GnRHa) test for ovaries), in order to define different enzymatic activities as entities not directly measurable *in vivo*. Subsequently, insulin resistance and secretion were assessed using the oral glucose tolerance test (oGTT) and homeostasis model assessment (HOMA) to define the possible effects of metformin on these parameters.

Subjects and methods

Patients

The study group consisted of 24 oligo/amenorrhoeic women with PCOS matching National Institute of Health (NIH) criteria (22), all with the clinical manifestation of hyperandrogenaemia such as hirsutism and/or acne and with an elevated free testosterone index. Vaginal ultrasonography was performed, with polycystic ovaries being found in 17 of the patients (71%), multifollicular ovaries (multiple subcortical follicles with no increase in the volume of the ovarian stromal area) in five patients (criteria according to Adams *et al.* (23)), and ovaries sonographically of normal appearance in two cases. Thyroid dysfunction, hyperprolactinaemia, hypercortisolism and the late onset of congenital adrenal hyperplasia were all excluded using the appropriate tests. None of the patients had taken oral contraceptives or any other steroid medication during the preceding 3 months. The age and body mass index (BMI) of the patients were 21.6 ± 4.8 years and $26.4 \pm 5.1 \text{ kg/m}^2$ (means $\pm$ s.d.) respectively. The control group consisted of 11 healthy women with regular menstrual cycles (28–33 days), 27.5 ± 3.5 years old, with BMI $22.3 \pm 3.0 \text{ kg/m}^2$ who also had not used oral contraceptives for at least the preceding 3 months, and who were without either clinical or laboratory signs of hyperandrogenaemia. The controls were recruited from among the healthcare personnel of the Institute of Endocrinology in Prague and their acquaintances.

Study protocol

The local ethics committee of the Institute of Endocrinology approved the protocol for the study. The patients were evaluated in the clinical department of the Institute of Endocrinology as outpatients, and after signing written informed consent underwent the following three tests in the early follicular phase of the same menstrual cycle (1st to 6th days).

ACTH test Synacthen (250 µg; CIBA-Geigy, Basel, Switzerland) was given i.m. between 0730 and 0830 h. Basal blood samples and samples withdrawn at 30 and 60 min were taken for the determination of testosterone, androstenedione, dehydroepiandrosterone (DHEA), DHEAS, 17-hydroxyprogesterone (17OHP), 17-hydroxypregnenolone (17OHPL) and cortisol.

GnRHa test Buserelin (100 µg; Suprefact; Hoechst Marion Roussel GmbH, Wein, Austria) was given s.c. after pretreatment with 2 mg dexamethasone at 2200 h the night before. Blood samples were collected between 0730 and 0800 h (basal), and after 4 and 24 h. The concentrations of testosterone, androstenedione, DHEA, oestradiol (E2), 17OHP, 17OHPL, SHBG, cortisol and LH were measured in all the samples.

oGTT with dextrose (75 g) After overnight fasting, blood samples were withdrawn for the determination of glucose, insulin and C peptide before drug administration, and then again after 30, 60, 120 and 180 min. Proinsulin (PI) was determined at 0, 30 and 180 min.

After the tests, the treatment started with metformin (Siofor Berlin Chemie) at 500 mg daily for 1 week. As the tolerance was good in all cases, the dose was increased to 1000 mg per day. This dose was chosen to minimize the possible side-effects of metformin. Patients were advised to follow an isocaloric diet during the study. After completing the course of treatment (27 ± 4 weeks), all the tests mentioned above were performed again in the same order. The pattern of the menstrual cycle was defined as follows: secondary amenorrhoea – absence of menstruation for 180 days or more; oligomenorrhoea – average length of the cycle between 42 and 180 days; regular cycle – average length of the cycle between 22 and 41 days, with no more than a 5-day variation from one cycle to another.

Laboratory assays

C peptide was measured by immunoradiometric assay (IRMA) kit from Immunotech (Marseilles, France). The cross-reactivity of antiserum with both insulin and glucagon was less than 0.05%. Glucose was evaluated in venous plasma (glucoso-oxidase method; Beckmann analyzer, Fullerton, CA, USA). Insulin was estimated by IRMA kit from Immunotech. PI was determined using an enzyme-linked immunosorbent assay (ELISA) kit

(IBL, Hamburg, Germany), with no cross-reactivity with insulin and with C peptide. DHEA and DHEAS were measured using radioimmunoassay (RIA) kits from Immunotech. DHEA was measured in diethyl-ether extracts, while DHEAS was determined directly in serum. Testosterone was determined by standard RIA using anti-testosterone-3-carboxymethyloxime:bovine serum albumin (BSA) antiserum and testosterone-3-carboxymethyloxime-tyrosylmethylester- ^{125}I as a tracer; the intra-assay and interassay coefficients of variation (CV values) were 7.2% and 10% respectively, and the sensitivity was 0.21 nmol/l. Androstenedione was determined using a standard RIA with anti-androstenedione-6-carboxymethyloxime:BSA antiserum and ^3H androstenedione as a tracer; the intra-assay and interassay CV values were 8.1% and 10.2% respectively, and the sensitivity was 0.39 nmol/l. 17OHPL was measured using a method published elsewhere (24). 17OHP, LH and follicle-stimulating hormone were measured by RIA kits from Immunotech. E2 was measured by the RIA kit from Immunotech. SHBG was estimated using the IRMA method (Orion, Espoo, Finland). Cortisol was measured by standard RIA using anti-cortisol-3-carboxymethyloxime:BSA antiserum and cortisol-3-carboxymethyloxime-TME- ^{125}I as a tracer; intra-assay and interassay CV values were 5.2% and 7.6% respectively, and the sensitivity was 12.3 nmol/l.

Computations

To evaluate the changes in adrenal and ovarian activity of enzymes acting in steroid metabolism, product/precursor ratios of the basal steroid levels and absolute levels after stimulation by ACTH or by GnRHa buserelin was employed. Considering the insignificance of the changes in steroid basal values after metformin treatment we used absolute values after ACTH stimulation instead of the differences between basal and stimulated values to avoid the inclusion of superfluous unexplained variability to statistical analysis. Androstenedione/DHEA and 17OHP/17OHPL were used as indices of 3β -hydroxysteroid dehydrogenase (3β -HSD) in C_{19} and C_{21} steroids respectively. Similarly, the androstenedione/17OHP and DHEA/17OHPL ratio was employed as an index of $\text{C}_{17,20}$ -lyase activity in the Δ^4 and Δ^5 pathways respectively. The testosterone/androstenedione ratio was used as an index of 17β -hydroxysteroid dehydrogenase (17β -HSD), and the E2/testosterone ratio as an index of aromatase (AR) activity. The DHEA/DHEAS ratio was used for the evaluation of the changes in equilibrium between DHEA and DHEAS. The index of free testosterone was calculated as $100 \times \text{testosterone}/\text{SHBG}$. For evaluation of insulin secretion and insulin resistance, HOMA indices were employed (HOMA-R for insulin resistance and HOMA-F for β cell function), as reported by Matthews *et al.* (25). Good correlations were found

with insulin sensitivity determined by euglycaemic clamp as a gold standard (25). The validation of insulin secretion was acceptable (26). The basal C protein/insulin molar ratio was used to evaluate the hepatic extraction of insulin.

Statistical treatment of the data

Two-way ANOVA was used for the evaluation of changes in the steroids and in the indices of enzyme activity. The treatment (before treatment, after treatment) and the time of the appropriate test were the first and second factor respectively. To attain an approximately Gaussian distribution of the data, which is a prerequisite for a correct ANOVA test, the variables were treated by logarithmic or power transformation to minimum skewness and the mean values within lower and upper limits of 95% CI values were retransformed to the original scale. For the evaluation of the change after metformin treatment within the individual stages of a dynamic test, Wilcoxon's paired test was used, because of the mostly non-Gaussian distribution of the data. Wilcoxon's paired test was also used to evaluate changes in HOMA-R, HOMA-F and BMI. The Mann-Whitney U test was used to compare the patients with the healthy control group. Null hypotheses of statistical test were evaluated on the 95% level of significance ($P < 0.05$).

Results

Clinical observations

The basal clinical characteristics and steroid levels for both the healthy controls and the patients with PCOS are given in Table 1. The mean age of the healthy controls was significantly higher ($P = 0.003$) than that of the PCOS patients. A tendency towards a higher mean BMI in the PCOS patients was found, but this tendency did not attain statistical significance.

After treatment with metformin, no significant change in BMI was observed (from 25.9 ± 5.9 to $25.5 \pm 5.4 \text{ kg/m}^2$; means $\pm$ s.d.). In 14 women (58%), there was a marked improvement of regularity in the menstrual cycle, defined either as resolution of secondary amenorrhoea or as a shortening of the average length of cycles to 35 or less days. In nine women (37.5%), there was no change in the average length of cycles. In one patient, secondary amenorrhoea developed during the treatment. In the group with the improved cycle, in eight patients the cycle during treatment was regular. Of these eight patients, two had suffered with secondary amenorrhoea and six with oligomenorrhoea before treatment. The rest of the group with improvement of the cycle (six patients) continued to exhibit irregular cycles despite the treatment; however, the length of the cycle was considerably shifted towards a normal duration. In

Table 1 Basal steroid values in patients with PCOS and healthy controls. Values are means $\pm$ s.d.

Variable	Women with PCOS (n = 24)	Healthy controls (n = 11)	P
Age	21.6 $\pm$ 4.8	27.5 $\pm$ 3.5	<0.004
BMI	26.4 $\pm$ 5.1	22.3 $\pm$ 3.0	NS
Cortisol (nmol/l)	454 $\pm$ 204	420 $\pm$ 123	NS
Testosterone (nmol/l)	2.35 $\pm$ 0.95	1.75 $\pm$ 0.49	NS
SHBG (nmol/l)	24.5 $\pm$ 8.8	54.7 $\pm$ 22.0	<0.007
Index of free testosterone	10.80 $\pm$ 5.25	4.11 $\pm$ 2.72	<0.005
Androstenedione (nmol/l)	5.4 $\pm$ 2.4	4.10 $\pm$ 1.77	NS
DHEA-S (μ mol/l)	8.08 $\pm$ 3.0	5.44 $\pm$ 2.08	<0.01
DHEA (nmol/l)	27.6 $\pm$ 13.8	19.6 $\pm$ 7.3	NS
17-OHP (nmol/l)	2.16 $\pm$ 1.32	1.59 $\pm$ 1.19	NS
17-OHPI (nmol/l)	13.2 $\pm$ 12.55	6.51 $\pm$ 5.31	NS

NS, not significant.

two of these patients, secondary amenorrhoea and in four patients oligomenorrhoea was found before the therapy. In the group of nine women with no change in the pattern of the cycle after therapy, in two cases secondary amenorrhoea, in four cases oligomenorrhoea and in two cases regular cycles were found before the treatment period. The last woman from this group with oligomenorrhoea underwent insertion of an intrauterine device during the treatment with metformin.

Basal steroid values

No significant differences in the levels of DHEA, DHEAS, 17OHP, 17OHPI and testosterone before and after treatment with metformin were found. Only the elevations of basal cortisol ($P < 0.07$, Wilcoxon's paired test) and androstenedione ($P < 0.06$, Wilcoxon's paired test) levels after metformin treatment approached statistical significance (Fig. 1). An insignificant increasing tendency in the levels of SHBG (from 24.8 ± 9.0 to 30.4 ± 15.7 nmol/l) was observed.

Adrenal activity (ACTH test)

No significant differences were found between the absolute stimulated values of cortisol, DHEA, DHEAS, 17OHP, 17OHPI, androstenedione and testosterone before and after treatment with metformin (Fig. 1). Nevertheless, when the enzyme activities were tested (Fig. 2) a significant decrease was detected in the stimulated activity of 3β -HSD in C_{21} steroids ($P < 0.05$, F-test, ANOVA). In addition, an increase in the activity of $C_{17,20}$ -lyase in the Δ^4 pathway ($P < 0.01$, F-test, ANOVA) was noted. A significant decrease in the activity of 17β -HSD was found ($P < 0.01$, F-test, ANOVA), while no changes were observed in the activity of 3β -HSD in C_{19} steroids and $C_{17,20}$ -lyase in the Δ^5 pathway. Finally, a significant growth in the DHEA/DHEAS ratio was found after metformin treatment ($P < 0.05$, F-test, ANOVA), particularly at the 60th minute of ACTH test values, where

the difference was again detected by means of Wilcoxon's paired test ($P < 0.05$). No significant changes were observed in the stimulated activities of 3β -HSD in C_{21} steroids and in $C_{17,20}$ -lyase in the Δ^4 pathway (results not shown).

Ovarian activity (GnRHa test with buserelin)

Significant decreases in the stimulated absolute levels of testosterone ($P < 0.05$, F-test, ANOVA), free testosterone index ($P < 0.01$, F-test, ANOVA), LH ($P < 0.05$, F-test, ANOVA) and E2 ($P < 0.01$, F-test, ANOVA), and an increase in the levels of 17OHPI ($P < 0.05$, F-test, ANOVA) were detected. No significant changes were found in the absolute concentrations of SHBG, 17OHP, DHEA, DHEAS and androstenedione (Fig. 3). In the indices of enzyme activities (Fig. 4), statistically significant decreases were observed in 3β -HSD in C_{21} steroids ($P < 0.01$, F-test, ANOVA), in $C_{17,20}$ -lyase in the Δ^5 pathway ($P < 0.01$) and in 17β -HSD ($P < 0.05$, F-test, ANOVA). The decrease in AR activity at the 24th hour of the buserelin test after metformin treatment was close to statistical significance ($P < 0.054$, Wilcoxon's paired test). No change was found in either 3β -HSD activity in C_{19} steroids or on $C_{17,20}$ -lyase activity in the Δ^4 pathway (results not shown).

Glucose tolerance (oGTT)

All of the patients but one initially had normal glucose tolerance as defined by the American Diabetes Association/World Health Organization criteria. After metformin treatment, the changes in PI and insulin were not significant: in insulin, from 11.57 (7.1; 18.0) μ IU/l to 8.6 (5.1; 13.7) μ IU/l (values are given as means; lower limit of 95% CI, upper limit of 95% CI). A significant elevation of stimulated C peptide levels in the 120th min (from 2.47 (2.11; 2.87) nmol/l to 3.43 (2.96; 3.94) nmol/l) after treatment was found ($P < 0.01$, F-test, ANOVA). Changes in the insulin/PI ratios were

Figure 1 Steroid changes and the index of free testosterone after metformin treatment in the ACTH test. Empty and hatched columns represent the retransformed mean values before and after treatment respectively. Vertical lines denote 95% CI values of retransformed mean values. Significance was evaluated by two-way ANOVA with treatment and ACTH test stage as the first and second factors respectively.

Figure 2 Changes in adrenal enzyme activity after metformin treatment in the ACTH test. Empty and stippled columns represent the retransformed mean values before and after treatment respectively. Vertical lines denote 95% CI values of retransformed mean values. Significance was evaluated by two-way ANOVA with treatment and ACTH test stage as the first and second factors respectively. * $P < 0.05$, ** $P < 0.01$, significance of the change after treatment with metformin within the ACTH test as a whole. + $P < 0.05$, significance of the change after treatment with metformin in the individual stages of the ACTH test. A, androstenedione; T, testosterone.

insignificant using ANOVA except significantly reduced values in the basal insulin/PI ratio after metformin treatment (from 6.72 (4.81; 9.2) to 4.1 (2.87; 5.69), $P < 0.05$, Wilcoxon's paired test). Changes in the basal C peptide/insulin molar ratios were insignificant using ANOVA, albeit a trend towards an increase could be seen (from 11.15 (8.36; 15.35) to 15.35 (11.15; 19.34)). No significant change was detected either in blood glucose or in insulin/glucose and PI/glucose ratios (not shown). No significant changes were observed after metformin treatment in either of the HOMA indices, although a trend towards a decline in HOMA-R was detected (from 2.31 (1.82; 2.92) to 1.60 (1.26; 2.03)).

Discussion

The aim of this study was to investigate changes in basal steroid levels and stimulated adrenal and ovarian

steroidogenesis after metformin treatment in women with PCOS. To define PCOS, the broader definition proposed by the NIH consensus in 1990 was used – chronic anovulation and hyperandrogenaemia. Our group under study consisted of young, only mildly obese patients with normal glucose tolerance.

The healthy controls were significantly older than the subjects, but this difference probably has no biological importance in this situation, as both groups were between 20 and 30 years of age.

In nearly two thirds of patients a marked improvement in the menstrual cycle pattern was seen. No significant change was found in basal steroid levels after metformin treatment; only basal cortisol and androstenedione levels displayed a tendency towards higher values. In not finding a change in the basal levels of testosterone, free testosterone or androstenedione after metformin treatment, this study therefore accords with the results obtained by some authors (19,

Figure 3 Steroid changes and the index of free testosterone after metformin treatment in the buserelin test. Empty and hatched columns represent the retransformed mean values before and after treatment respectively. Vertical lines denote 95% CI values of retransformed mean values. * $P < 0.05$, ** $P < 0.01$, significance of the change after treatment with metformin in the buserelin test as a whole. Significance was evaluated by two-way ANOVA with treatment and buserelin test stage as the first and second factors respectively. + $P < 0.05$, +++ $P < 0.001$, significance of the change after treatment with metformin in the individual stages of the buserelin test.

Figure 4 Changes in ovarian enzyme activity after metformin treatment in the buserelin test. Empty and dotted columns represent the retransformed mean values before and after treatment respectively. Vertical lines denote 95% CI values of retransformed mean values. Significance was evaluated by two-way ANOVA with treatment and the buserelin test stage as the first and second factors respectively. * $P < 0.05$, ** $P < 0.01$, significance of the change after treatment with metformin in the buserelin test as a whole. + $P < 0.05$, ++ $P < 0.001$, significance of the change after treatment with metformin in the individual stages of the buserelin test. A, androstenedione; T, testosterone.

20), although not with those obtained by others (12–15). Most of these studies found a decrease only in free testosterone, without concomitant changes in other androgens. This may be explained by an elevation of SHBG after metformin treatment (possibly via reduction of hepatic insulin resistance). Duration of treatment and the dose of metformin can probably play a role. Only a few studies in PCOS (15, 18, 19, 27–29) lasted for 6 months or longer, and in one (19) the significant fall in androgen levels was found only after 6 weeks but not after 6 months of treatment.

As a next step, evaluation of enzymatic activities after stimulation of the adrenals and ovaries was attempted. Basal levels of androgens are the product of both ovaries and adrenals and it is difficult to distinguish the source of hormonal disturbance. PCOS is commonly connected with ovarian overproduction of 17OHP and androstenedione and with hypersecretion of these steroids after GnRHa challenge (3). In addition, an adrenal steroid excess with hypersecretion of androstenedione and DHEA after ACTH is frequently found in PCOS. The elevation of ACTH-stimulated enzymatic activities was not always connected with the elevation of these activities in the basal state (30). As the lowering of androgens is not equivocally found after metformin, we supposed that this observation could be

due to more subtle effects of metformin on the stimulated steroid secretion (maybe not necessarily reflected by the lowering of basal levels).

The manifestation of the changes in enzyme activity on the steroidogenic pathways was more pronounced after challenge with trophic hormones compared with the basal values.

We are aware of only two studies concerning the effects of metformin on adrenal steroidogenesis in women with PCOS, with discrepant results. After 1 month of metformin treatment, la Marca *et al.* (11) found a significant reduction in the maximum increment and area under the curve of 17OHP, androstenedione, testosterone and free testosterone after ACTH stimulation, and decreases in the 17OHP/progesterone ratio (an index of C17 hydroxylase activity) and androstenedione/17OHP ratio (an index of the activity of C17,20 lyase in the $\Delta 4$ pathway). On the other hand, Unluhizarci *et al.* (10) found no difference in 17OHP or androstenedione response after ACTH stimulation, after a 3-month course of metformin. In our study, no significant changes in their absolute values were found, in terms of ACTH-stimulated steroid levels after metformin treatment. However, examination of enzymatic activities defined as product/precursor ratios revealed a significant decrease in the stimulated

activity of 3β -HSD in C_{21} steroids, an increase in the activity of C17,20-lyase in the $\Delta 4$ pathway and a significant decrease in the activity of 17β -HSD.

The finding of increased C17,20-lyase activity in the $\Delta 4$ pathway was surprising. It contradicts a study (11) which found a decrease in its activity after metformin treatment. Unfortunately, it was not possible to examine the activity of C17,20 hydroxylase in both ($\Delta 4$ and $\Delta 5$) pathways. It cannot be excluded that such activity decreases, leading in turn to a decreased concentration of 17OHP. This situation might then be interpreted as an increase in the activity of the next enzymatic step (lyase). A decrease in 17β -HSD activity leads to a decrease in testosterone production (as the most active androgen).

There was a tendency towards lower levels of DHEAS after metformin treatment, not reaching statistical significance. A significant increase was then found in the DHEA/DHEAS ratio. This might be due either to the lower synthesis of DHEAS or to changes in the interconversion of these two steroids. A recently published study (16) has described a decline in DHEAS after metformin only in a subgroup of patients with high basal DHEAS. This subgroup also had a better clinical outcome (defined as an improvement in the length of the menstrual cycle) than in the subgroup with initially normal DHEAS.

With regard to ovarian steroid production, significant reductions in stimulated levels of 17OHP, testosterone, the index of free testosterone, E2 and LH were found. These findings again accord with some studies (12–14, 18) but not with others (20, 30). In steroid enzyme activities, decreases in the stimulated activity of 3β -HSD in C_{21} steroids, in the activity of C17,20-lyase in the $\Delta 5$ pathway, in 17β -HSD and in androstenedione activity were found.

Examining glycidic metabolism by oGTT, we found a significant increase in the stimulated C peptide levels, without significant changes in either glucose levels or insulinaemia, and without significant change in the basal C peptide/insulin ratio (as an index of hepatic insulin clearance). In the literature, information is scarce regarding C peptide secretion after metformin treatment in groups of patients with normal glucose tolerance and insulin resistance (as women with PCOS mostly have). We are aware of a study (31) finding no change in either basal or stimulated levels of C peptide in a group of hypertensive patients after metformin. In a group of obese type 2 diabetics an increase in C peptide levels after metformin treatment was found (32). These discrepancies are difficult to explain and deserve further research. In this study, no significant changes were found in HOMA-R and fasting insulin, as commonly used indices of insulin resistance, or in HOMA-F as an index of insulin secretion. Nevertheless, a slight tendency to reduction in fasting insulinaemia and HOMA-R was seen. The gold standard for estimate of insulin resistance is the euglycaemic clamp. To our

knowledge, there have been only a few studies using the clamp in PCOS women under metformin treatment (15, 18). In the study of Diamanti-Kandarakis *et al.* (15), an increase in insulin sensitivity after metformin not accompanied by a significant decrease in fasting insulin was reported. Several studies were conducted with metformin in type 2 diabetic patients (33, 34). These studies reported a relatively small improvement in peripheral insulin sensitivity and the authors suggested the suppression of endogenous hepatic glucose production as the primary mechanism of metformin action. Concerning the hyperbolic relationship of fasting insulin and insulin sensitivity (35), we suggest that the relatively small improvement in insulin sensitivity cannot induce an apparent fall in fasting insulin that could reach statistical significance. The same probably holds for HOMA-R as it is derived from basal insulinaemia. Another mechanism responsible for the fall in basal insulin levels is the improvement in hepatic insulin clearance. As previously, we observed only a small improvement. Other studies dealing with PCOS and finding a significant decrease in fasting insulin levels almost invariably involved women with an average BMI between 27 and 36 kg/m² (10, 16, 18, 19, 27). Moreover, the degree of hyperinsulinaemia in these studies was greater than in our patients.

In conclusion, the following can be stated. (1) Nearly two thirds (58%) of the patients reported an improvement in the regularity of their menstrual cycle, and only in one patient did the menstrual pattern worsen during treatment. (2) The long-term course of metformin treatment in women with PCOS reduced activity in several of the metabolic pathway enzymes responsible for androgen production in both the ovaries and the adrenals. (3) The methods used to detect insulin resistance (HOMA model and basal insulin) did not discover a significant improvement after metformin treatment; nevertheless, a reduction in insulin resistance after metformin cannot be excluded – using more sophisticated methods of detection. (4) Metformin is a clinically useful drug in the treatment of PCOS (even at the dose of 1000 mg/day), with good tolerance.

Acknowledgements

This work was supported by research grants nos 4847-3, 6702 and 5398-3 of the Internal Grant Agency of the Czech Ministry of Health, and by research design no. 000000023761 of the Institute of Endocrinology in Prague.

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Received 2 October 2000

Accepted 16 February 2001