Impaired insulin-dependent glucose metabolism in granulosa-lutein cells from anovulatory women with polycystic ovaries

S.Rice\textsuperscript{1,4}, N.Christoforidis\textsuperscript{2}, C.Gadd\textsuperscript{2}, D.Nikolaou\textsuperscript{2}, L.Seyani\textsuperscript{3}, A.Donaldson\textsuperscript{3}, R.Margara\textsuperscript{2}, K.Hardy\textsuperscript{1} and S.Franks\textsuperscript{1,5}

\textsuperscript{1}Institute of Reproductive and Developmental Biology, \textsuperscript{2}Department of Obstetrics and Gynaecology, Imperial College London, London and \textsuperscript{3}Clinical Chemistry Laboratory, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
\textsuperscript{4}Present address: Department of Basic Medical Sciences, St George’s Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE, UK
\textsuperscript{5}To whom correspondence should be addressed. E-mail: s.franks@imperial.ac.uk

BACKGROUND: Insulin resistance and hyperinsulinaemia are well-recognized characteristics of anovulatory women with polycystic ovary syndrome (PCOS) but, paradoxically, steroidogenesis by PCOS granulosa cells remains responsive to insulin. The hypothesis to be tested in this study is that insulin resistance in the ovary is confined to the metabolic effects of insulin (i.e. glucose uptake and metabolism), whereas the steroidogenic action of insulin remains intact. METHODS: Granulosa-lutein cells were obtained during IVF cycles from seven women with normal ovaries, six ovulatory women with PCO (ovPCO) and seven anovulatory women with PCO (anovPCO). Mean body mass index was in the normal range in all three groups. Granulosa-lutein cells were cultured with insulin (1, 10, 100 and 1000 ng/ml) and LH (1, 2.5 and 5 ng/ml). Media were sampled at 24 and 48 h and analysed for glucose uptake, lactate production and (48 h only) progesterone production. RESULTS: Insulin-stimulated glucose uptake by cells from anovPCO was attenuated at higher doses of insulin (100 and 1000 ng/ml) compared with that by cells from either ovPCO (\(P < 0.02\)) or controls (\(P < 0.02\)). Insulin and LH stimulated lactate production in a dose-dependent manner, but insulin-dependent lactate production was markedly impaired in granulosa-lutein cells from anovPCO compared with either normal (\(P < 0.002\)) or ovPCO (\(P < 0.0001\)). By contrast, there was no difference in insulin-stimulated progesterone production between granulosa-lutein cells from the three ovarian types. CONCLUSIONS: Granulosa-lutein cells from women with anovPCOS are relatively resistant to the effects of insulin-stimulated glucose uptake and utilization compared with those from normal and ovPCO, whilst maintaining normal steroidogenic output in response to physiological doses of insulin. These studies support the probability of a post-receptor, signalling pathway-specific impairment of insulin action in PCOS.

Key words: granulosa cells/insulin resistance/LH/polycystic ovaries/progesterone

Introduction
Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in premenopausal women and is the main cause of anovulatory infertility (Franks, 1995). The aetiology of anovulation remains unknown, but hyperinsulinaemia and insulin resistance, which are common characteristics of anovulatory women with PCOS (Book and Dunai, 1999) and are associated with an increased risk of type 2 diabetes in later life (Legro et al., 1999), may play a role in the mechanism of anovulation (Venkatesan et al., 2001). Paradoxically, despite clear evidence for peripheral insulin resistance in PCOS (Book and Dunai, 1999), granulosa cells from anovulatory women with PCOS remain responsive to the steroidogenic action of insulin. The effects of a submaximal dose of insulin on estradiol (E\textsubscript{2}) and progesterone production are similar to those in granulosa cells from women with normal ovaries (Willis et al., 1996). Insulin has been shown to act through its own receptor to modulate steroidogenesis, and responsiveness to LH, in human granulosa cells from normal and polycystic ovaries (Willis and Franks, 1995; Willis et al., 1996; 1998).

Insulin sensitivity is conventionally defined in terms of glucose utilization by peripheral tissues, notably adipose tissue and muscle. Glucose uptake and metabolism by granulosa cells play an important part in making energy substrate available to the maturing oocyte (Flint and Denton, 1969; Donahue and Stern, 1968; Leese and Barton, 1984; Hillier et al., 1985; Downs and Utech, 1999; Roy...
and Terada, 1999). The role of gonadotrophins in regulation of glucose metabolism has been described (Flint and Denton, 1969; Hillier et al., 1985; Zuelke and Brackett, 1992; Roy and Terada, 1999; Roberts et al., 2004), but very little is known about the effects of insulin in granulosa cells. Preliminary data from studies of human granulosa-lutein cells have suggested that there is an impairment of insulin-mediated lactate production in PCOS (Lin et al., 1997; Fedorcsak et al., 2000), providing support for the hypothesis that there is a selective abnormality in post-receptor insulin signalling affecting glucose uptake and metabolism. These studies did not, however, include measurement of glucose uptake, which is a direct target of insulin action. Progesterone production in normal granulosa-lutein cells from both normal and polycystic ovaries (Fedorcsak et al., 2000), is a direct target of insulin action. Progesterone production in PCOS (Lin et al., 2004), but very little is known about the effects of insulin in granulosa-lutein cells from both normal and polycystic ovaries (Fedorcsak et al., 2000). Neither study included a group of women with polycystic ovaries and regular cycles, a population of patients in whom (in contrast to anovulatory women with PCO) we have previously reported near-normal insulin secretion and sensitivity (Robinson et al., 1993).

The aim of this study was therefore to test the hypothesis that insulin resistance does affect granulosa cells of the polycystic ovary but is restricted to the action of insulin on glucose uptake and metabolism. To this end we used granulosa-lutein cells from well-characterized women with ‘classic’, anovulatory PCOS, women with polycystic ovaries but who had a history of regular, ovulatory cycles, and a control group of women with normal ovaries and regular cycles. The experiments were designed to look at the effects of physiological doses of insulin and LH on carbohydrate metabolism (i.e. glucose utilization and lactate production) in cultured granulosa-lutein cells from normal and polycystic ovaries. In addition, levels of progesterone produced by the same cells in response to insulin and LH were measured, in order to compare and contrast steroidogenesis and glucose metabolism in normal and polycystic ovaries.

**Patients and methods**

**Patients**

Granulosa-lutein cells were obtained, with informed, written consent, from follicular aspirates of 20 women [seven ovulatory subjects with normal ovaries, six with ovulatory polycystic ovaries (ovPCO) and seven with anovulatory polycystic ovaries (anovPCO)] undergoing treatment by IVF or ICSI for infertility at the Hammer smith Hospital, or the Chelsea and Westminster Hospital (one patient), London. Sample size was estimated on the basis of the previously published studies reporting differences in granulosa cell lactate production between anovPCOS and normal controls (Lin et al., 1997; Fedorcsak et al., 2000). Ethical approval for this study was obtained from the Hammersmith, Queen Charlotte’s and Chelsea Hospitals Research Ethics Committee, and the Riverside Research Ethics Committee. Subjects in the three groups were well matched for age and body mass index (BMI) (Table I), and in all but three women (one in each group) BMI was <25.

Polycystic ovaries were defined, using ultrasound, by the presence of two or more of the following criteria: enlarged ovarian volume (>9 ml), 10 or more follicles 2–10 mm in diameter, and increased density and volume of stroma (Adams et al., 1986; Polson et al., 1988). Patients with polycystic ovaries and a history of oligomenorrhea or amenorrhea were classified as anovulatory (anovPCO) and those with regular menstrual cycles (cycle length between 21 and 35 days with no more than a 4 day variation between cycles in individual subjects) (Polson et al., 1988) were classified as ovulatory (ovPCO). All women with regular cycles had evidence of ovulation as judged by a mid-luteal progesterone concentration >25 nmol/l in a pretreatment ‘assessment’ cycle. The primary indications for IVF or ICSI were as follows. In the normal ovary group: male factor infertility (n = 5) and tubal disease (n = 2); in ovPCO: male factor infertility (n = 4), tubal disease (n = 1) and unexplained infertility (n = 1); in anovPCO: tubal disease (n = 3), anovulation resistant to induction of ovulation (n = 3) and persistent infertility despite successful induction of ovulation (n = 1).

**Blood samples and follicular fluid aspiration**

Peripheral venous blood was collected between days 2 and 6 of a pretreatment assessment cycle from patients with regular cycles, after spontaneous menses in women with anovPCO whose cycle length was <42 days, or at random in anovPCO subjects with oligo- or amenorrhea. An additional, fasting blood sample was taken (for glucose and insulin measurements only) at the time of egg collection for IVF. Serum levels of insulin, FSH, LH, sex-hormone binding globulin (SHBG), androstenedione, testosterone and E2 were measured by immunoassay using methods previously reported by this group (Polson et al., 1987; Robinson et al., 1992; White et al., 1995; Gilling-Smith et al., 1997). Insulin resistance (IR) was calculated using the homeostatic model assessment (HOMA) method [HOMA-IR = insulin/(22.5e−11[log Glucose])] (Matthews et al., 1985; Jayagopal et al., 2003). Pituitary-gonadal suppression was achieved by the administration of a GnRH analogue Buserelin (Suprecur; Shire Pharmaceuticals, Andover, UK). Following adequate suppression of pituitary-ovarian activity, superovulation of patients with recombinant FSH (rFSH) was performed using either Puregon (Organon, Oss, The Netherlands) or Gonal-F (Serono, Feltham, UK). The ovarian response was monitored by ultrasound and serum E2 levels. Once three or more follicles had reached a mean diameter of 17 mm and E2 levels of 3000 pmol/l were achieved, HCG (Profasi; Serono) 10000 IU was administered.
Trans-vaginal follicle aspiration was performed 32–36 h after HCG was given and the follicular fluid aspirates were collected in sterile, 75-ml cell culture flasks (Falcon; Becton Dickinson Labware, Oxford, UK).

Isolation and culture of granulosa-lutein cells

Oocytes were recovered and granulosa-lutein cells were then separated from the follicular fluid aspirates by centrifugation at 200 g for 5 min and re-suspended in phosphate-buffered saline (PBS) (Invitrogen, Paisley, UK). Red blood cell contamination was eliminated by layering the granulosa cells onto a 45% v/v Percoll™ gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifuging at 400 g for 20 min. The cells were then washed twice, re-suspended in Medium 199 (Invitrogen), and cell number and viability determined by Trypan Blue exclusion test. Granulosa-lutein cells were plated in 96-well cell culture plates (NUNC™; Becton Dickinson Labware) at a density of 2 × 10⁵ viable cells/well in 200 µl of Medium 199 supplemented with 1% v/v fetal bovine serum, antibiotics (penicillin and streptomycin) and 200 mmol/l l-glutamine (all from Invitrogen). Each experimental condition, in cells from each patient, was repeated in triplicate. After 48 h incubation at 37°C in 5% CO₂ the serum-containing medium was removed and the cells were washed with PBS. They were then incubated for a further 48 h with 200 µl serum-free Medium 199 with or without varying doses (1, 10, 100 and 1000 ng/ml) of human recombinant insulin (Roche Diagnostics Ltd, Lewes, UK) or recombinant LH (1, 2.5 and 5 ng/ml) (Serono International S.A., Geneva, Switzerland; kindly supplied for cell culture by Professor S.Hillier, University of Southampton, UK) (Willis et al., 1998). It is based on the conversion of MTS tetrazolium reagent by metabolically active cells into a coloured formazan product that is soluble in tissue culture medium. Once all the medium was removed from the wells at the end of the experiment (for steroid hormone analysis), 100 µl of pre-warmed and gassed Medium 199 was added to each well before the direct addition of 20 µl of CellTitre 96® AQ Green One Solution Reagent into each well. After 2 h incubation the absorbance is recorded at 490 nm with a 96-well plate reader, according to manufacturer’s instructions. The absorbance value, read from a standard curve, gives an indication of cell number.

Statistical analysis

Experiments to measure lactate, glucose and progesterone were done in triplicate wells for each treatment condition. Because there was considerable variability in basal values between subjects, the mean results from each patient were also expressed as a percentage of the control (i.e. basal, no treatment added) value at each time point. The results from all the patients within a particular group (i.e. normal, ovPCO and anovPCO) were then combined and expressed graphically as mean ± SEM. Statistical analysis was performed using the Instat and Prism software packages (Macintosh version 3.0, GraphPad Software, San Diego, CA, USA). Between-group comparisons were made using analysis of variance (ANOVA) with post-hoc correction for multiple comparisons. For baseline clinical and endocrine data, and for data relating to outcome of IVF/ICSI, between-group comparisons were made using one-way ANOVA and/or unpaired t-tests, as indicated in Results. A P-value of ≤ 0.05 was considered significant.

Results

Clinical and endocrine profiles of subjects

Baseline (pretreatment) clinical and endocrine data from the three groups of subjects are shown in Table I. Age and BMI did not differ significantly between the three groups. LH concentrations were higher (P = 0.05, unpaired, two-tailed
and those of SHBG were lower ($P = 0.03$) in anovPCO than in controls. There was no difference in the mean serum levels of FSH, androstenedione, testosterone and free androgen index (testosterone: SHBG × 100) between the three categories of subjects (Table I). Mean fasting glucose was very similar in all three categories. Fasting insulin levels were highest in the anovPCO group (three of the subjects had levels >10 mU/l) but were not significantly different between groups. The calculated HOMA-IR values in anovPCO were higher than in either ovPCO patients ($P = 0.04$) or controls (though not significantly; $P = 0.28$), indicating that patients in with anovPCO were, as expected (Robinson et al., 1993), relatively insulin resistant.

In terms of outcome of treatment, the total dose of FSH required for superovulation differed between groups ($P = 0.029$, one-way ANOVA) and was lower in the anovPCO groups than in normal subjects [1609 ± 220 versus 2796 ± 354 IU (mean ± SEM), $P < 0.05$, after Bonferroni correction for multiple comparisons]. Despite this, the number of small follicles (2–16 mm in diameter) was higher in both PCO groups than normal (ovPCO 16 ± 2.4, anovPCO 13 ± 2.3, normal 6 ± 0.8, $P = 0.0034$, ANOVA; ovPCO versus normal, $P < 0.01$; anovPCO versus normal, $P < 0.05$). There were no significant differences between the groups in peak serum $E_2$ concentrations, number of oocytes collected or the percentage of oocytes that were fertilized.

**Effects of insulin and LH on glucose metabolism and progesterone production in granulosa-lutein cells**

The uptake of glucose and production of lactate by granulosa-lutein cells was measured at 2, 24 and 48 h after addition of insulin or LH. There was no significant change in glucose uptake or lactate production with insulin or LH. The production of progesterone was increased in the presence of insulin or LH. The figure below shows the results of these experiments.

![Figure 1](image)

Figure 1. Glucose uptake, lactate production and progesterone production (mean ± 1 SD of absolute values) by cultured granulosa-lutein cells after 48 h of incubation in the presence of insulin (10 ng/ml) or LH (2.5 ng/ml) in seven women with normal ovaries, six with ovulatory polycystic ovaries (ovPCO) and seven with anovulatory polycystic ovaries (anovPCO). Open bars show baseline (no insulin or LH) values and shaded bars show responses to insulin or LH. Note large SDs, reflecting wide variation in baseline (and stimulated) values between individual subjects.
or lactate concentrations after 2 h (data not shown). Glucose uptake and lactate production, expressed as nmol/well/h, were similar at 24 and 48 h after addition of insulin or LH and so data are shown for the 48 h incubation only. Progesterone values presented are also those obtained after 48 h incubation. The results from the cell proliferation assay at the end of the experiments showed that there was no significant mitogenic activity of insulin and cell viability was >90% (data not shown).

Absolute values for glucose uptake, lactate production and progesterone accumulation (control and in response to a submaximal dose of insulin or LH) are summarized in Figure 1. There was considerable variability between individual subjects in terms of basal and stimulated glucose uptake, lactate production and progesterone accumulation. The results of the full dose–response curves are therefore presented in terms of percent change compared with baseline (i.e. control with no insulin or LH) (Figure 2).

**Glucose uptake**

The addition of both insulin and LH resulted in a dose-dependent increase in glucose uptake (Figure 2). In both normal and ovPCO groups, there was a linear increase in glucose uptake with increasing doses of insulin, but in cells from anovPCO, the response to higher doses was attenuated; there was no further increase in glucose production at insulin doses above 10 ng/ml. Although there was no significant difference in the overall dose–response curves between the three groups, the change in glucose uptake between 10 and 1000 ng/ml insulin was significantly different between the groups ($P = 0.02$, one-way ANOVA). The increase in glucose uptake was less in granulosa cells from anovPCO (mean $-13\%$) than in either normal (+22%; $P = 0.03$, unpaired t-test) or ovPCO (+39%; $P = 0.02$). LH-stimulated glucose uptake was similar in each of the groups, reaching a maximal response at a dose of 2.5 ng/ml in each group.

![Figure 2. Glucose uptake, lactate production and progesterone production (expressed as a percentage of baseline values) by cultured granulosa-lutein cells after 48 h of incubation in response to insulin (1–1000 ng/ml) or LH (1–5 ng/ml) in seven women with normal ovaries (open squares), six with ovPCO (open diamonds) and seven with anovPCO (filled circles). Individual data points indicate mean ± SEM. Glucose uptake in anovPCO cells was attenuated at insulin concentrations above 10 ng/ml (see Results). There were significant differences in the dose–response curves between the groups with respect to insulin-stimulated lactate accumulation ($P \leq 0.0001$ ANOVA) and LH-stimulated progesterone production ($P = 0.012$, ANOVA). Note the different scales for the figures of insulin- and LH-stimulated progesterone production.](image-url)
**Lactate production**

Insulin stimulated lactate production by granulosa-lutein cells from normal and ovulatory PCO subjects, in a dose-dependent manner. However, cells from the anovPCO subjects were significantly less responsive to insulin than those from both of the other two groups, so that only the higher, supra-physiological doses of insulin (100 and 1000 ng/ml) stimulated an increase in lactate production over baseline. Lactate production in the anovPCO group was significantly reduced compared with both normal \( P = 0.0002 \) (24h), \( P = 0.002 \) (48h), two-way ANOVA and ovPCO \( P < 0.0001, P < 0.0001 \) groups at both 24h (data not shown) and 48h, respectively. LH also, dose-dependently, stimulated lactate production in each of the three groups, but in contrast to the response to insulin, there was no significant difference in response between the groups (Figure 2).

**Progestosterone production**

Insulin stimulated progesterone in a dose-related manner in all three groups. The lowest dose of insulin (1 ng/ml) had no effect on progesterone accumulation in the cells from anovPCO, but there was no significant difference between the groups in dose–response curves.

LH stimulated progesterone production in all three groups with a peak response of two to three times that of the basal value (Figure 2). Progesterone production by the anovPCO cells was significantly greater than from the normal \( P = 0.046, \) ANOVA \( P = 0.03, \) ANOVA \( P = 0.0001 \) groups at both 24h (data not shown) and 48h, respectively. LH also, dose-dependently, stimulated lactate production in each of the three groups, but in contrast to the response to insulin, there was no significant difference in response between the groups (Figure 2).

**Discussion**

The major finding of this study is that insulin-stimulated lactate production by granulosa-lutein cells from women with anovPCO is significantly impaired compared with the response in cells obtained from women with either normal ovaries or from ovulatory women with polycystic ovaries. However, the steroidogenic response to physiological doses of insulin is maintained. These results are in marked contrast to the effects of LH: LH-induced glucose uptake and lactate production are similar in cells from anovPCO to those in the other two groups, whilst progesterone production in response to LH is significantly enhanced in anovPCO. Our data are consistent with the results of two previous reports of impaired lactate production in granulosa-lutein cells from women with PCOS (Lin et al., 1997; Fedorcsak et al., 2000). However, measurements of glucose uptake were not presented and neither study included cells from ovulatory women with polycystic ovaries. Progesterone production was measured in one of the two studies (Fedorcsak et al., 2000), but, rather unexpectedly, there was no response of progesterone to insulin stimulation in the control group, making it impossible to determine whether there was resistance to the steroidogenic action of insulin in PCOS. Our results are perhaps all the more remarkable in that, in contrast to the study by Fedorcsak et al. (2000), our patients with anovPCO were not obese and were not selected specifically on the basis of in-vivo evidence of insulin resistance (although they were more insulin resistant than the ovulatory groups). This is reflected in the observation that although HOMA-IR was higher in anovPCO than in either controls or ovPCO, the differences only reached statistical significance when women with anovPCO were compared with those with ovPCO. It is well known that the obesity amplifies the difference in insulin resistance between women with PCOS and weight-matched controls (Holte et al., 1994), so it is likely that the impairment of insulin sensitivity would be even more marked in obese anovulatory women. The anovPCO group included three subjects who were resistant to induction of ovulation; however, this is unlikely to have introduced a significant bias in the group. Although one might predict that these women would be more insulin resistant than the remainder of the group, there was in fact no difference in HOMA-IR between these subjects and those with other indications for IVF.

Lactate is the end point of glycolysis and therefore a suitable indicator of glucose utilization and carbohydrate metabolism. Previous investigations into energy metabolism of cultured ovarian follicles have shown that anaerobic glycolysis plays a major part in supplementing the ATP requirements of the growing follicle and oocyte. This is believed to be due to decreasing partial pressure of oxygen from the periphery to the centre of the avascular pre-antral follicle, although even upon antrum formation there is no reduction in lactate production (Boland et al., 1993). It is probable that granulosa cells show a preference for this pathway since cells cultured in monolayers also produce high concentrations of lactate (Billig et al., 1983; Hillier et al., 1985). Thus lactate production may be the most efficient route for generation of the large amounts of ATP required for follicle growth and steroidogenesis in a relatively anoxic environment (Hillier et al., 1985). Glucose uptake and metabolism in granulosa cells are known to be regulated by gonadotrophins (Flint and Denton, 1969; Allen et al., 1981; Hillier et al., 1985), but surprisingly little has been published on the effects of insulin on glucose metabolism in the ovarian follicle (Weber and La Barbera, 1988; Eppig et al., 2000).

Previous studies have shown that the lactate concentration in follicular fluid is considerably higher than in plasma, indicating that most of it is derived from granulosa cell glycolysis of glucose (Leese and Lenton, 1990). The observed impairment of insulin-mediated lactate accumulation is likely to represent both attenuated glucose uptake and a reduction in glycolytic activity in cells from anovPCO, which, in vivo, may result in a reduction of the energy supplied to the cells for their growth and proliferation. Glucose availability has been directly linked to follicle growth and rate of ovulation (Downing et al., 1995); growth of isolated, murine, antral follicles does not progress in its absence (Boland et al., 1994). Antral follicles have been shown to use predominantly glycolytic methods of energy production (Boland et al., 1994) with the end product, lactate, increasing with follicle diameter (Harlow et al., 1987). Importantly, we found no difference in LH-stimulated lactate production between the normal, ovPCO and anovPCO groups, showing a specific defect in the insulin-mediated pathway. It is unclear how LH exerts its effects on glucose uptake and metabolism, but it
has been shown to have a direct action on the activities of the enzymes in the glycolytic pathway (Roy and Terada, 1999). In addition, LH acting through its own receptor has also been shown to stimulate tyrosine phosphorylation (Sekar and Veldhuis, 2001), an effect that is augmented by the addition of insulin (Carvalho et al., 2003). This cross-talk between the signaling pathways may explain the interaction between insulin and LH that is important in PCOS (Willis et al., 1996).

Insulin-mediated glucose uptake by cultured granulosa-lutein cells was not dramatically affected by ovarian status, but there was nevertheless a significant attenuation of glucose uptake in response the highest doses of insulin in cells from anovPCO. One possible explanation for this phenomenon is that, in granulosa-lutein cells from anovPCO, there is either limited availability or impaired mobilization of the glucose transporter protein GLUT-4. Little is known about glucose transport in granulosa cells. GLUT-4 is one of a family of facilitative glucose transport proteins, and is responsible for acute insulin-stimulated uptake of glucose (Burlant et al., 1991). Its expression is largely restricted to insulin-sensitive tissues such as skeletal muscle, adipose tissue and cardiac muscle, though GLUT-4 proteins have been detected in both granulosa and theca compartments of large (2–8 mm) follicles in sheep (Williams et al., 2001). Adipose and skeletal muscle tissues, taken from subjects from insulin resistant states (PCOS and type 2 diabetes, respectively), have been shown to have a reduced GLUT-4 content (Rosenbaum et al., 1993; Gaster et al., 2001). We have demonstrated, in preliminary studies, expression of GLUT-4 protein in murine granulosa cells (Roberts et al., 2004) and mRNA using RT–PCR in human granulosa-lutein cells (unpublished observations). As yet, however, no information is available regarding the cellular content of GLUT-4 protein, or its translocation to the cell membrane, in granulosa cells from polycystic ovaries.

Notwithstanding the observation of attenuated glucose uptake in anovPCO granulosa cells, the differences between anovPCO and the other groups in terms of insulin-stimulated lactate production are much more obvious than those in glucose uptake, suggesting that the impact of insulin resistance in granulosa cells of PCOS is greater on glycolysis than it is on glucose uptake. This is consistent with previous observations that suggest that the major route of glucose metabolism in granulosa cells is via the glycolytic pathway (Boland et al., 1993; Cetica et al., 2002).

It has been proposed that insulin resistance in PCOS is both tissue-specific and (within the same tissue) signalling pathway-specific (Venkatesan et al., 2001). The disparity between the attenuated insulin-stimulated lactate production and the unimpaired steroidogenic response to insulin observed in this study supports the view that there may be selective insulin resistance in anovulatory PCOS that is distal to insulin binding to its receptor, i.e. signalling pathway-specific. This is consistent with the results of studies of insulin action in skin fibroblasts from women with PCOS (Book and Dunai, 1999). In these studies, glucose incorporation into glycogen, representing the metabolic action of insulin, was impaired, whilst the mitogenic effect of insulin was unaffected. Interestingly, in this context, Wu et al. (2003) recently described a selective impairment of insulin-stimulated glucose incorporation into glycogen, in granulosa cells from women with PCOS. The concept that insulin resistance in PCOS is selective in terms of which specific post-receptor signaling pathway is affected is supported by further studies, both in vitro and in vivo, which have shown that, in PCOS, the metabolic actions of insulin in adipocytes and muscle (as previously shown for fibroblasts) are impaired but the mitogenic actions remain intact (Venkatesan et al., 2001). Pathway-specific insulin resistance is not, however, confined to PCOS; it has also been described in type 2 diabetes (Goodyear et al., 1995; Hunter and Garvey, 1998). Little is known, however, about whether the steroidogenic effects of insulin are impaired in PCOS. Data from this and a previous study (Willis et al., 1996) suggest that insulin-stimulated production of E2 and progesterone by isolated granulosa or granulosa-lutein cells from anovulatory women with PCOS does not differ significantly from that in cells from normal ovaries. It is not entirely clear which post-receptor signalling pathway is utilized in insulin-mediated steroidogenesis, but signalling by both the mitogen-activated kinase and the phosphotyrosine phosphatase 3-kinase (PI3-K) pathways are thought to be involved (Wood and Strauss, 2002).

The precise mechanism of selective insulin resistance in PCOS remains uncertain. There is evidence for enhanced serine phosphorylation of the insulin receptor and downstream signalling proteins, which is associated with decreased insulin receptor (tyrosine) autophosphorylation. Inhibitors of serine phosphorylation are able to reverse the defect in tyrosine phosphorylation, suggesting that excess serine kinase activity is the primary, or predisposing, phenomenon (Dunaif et al., 1995; Venkatesan et al., 2001).

The insulin receptor substrates IRS-1 and IRS-2, which play a key role in directing post-receptor signalling, may be implicated in pathway-specific insulin resistance. Tyrosine phosphorylation of IRS-1 and -2 leads to binding to the Src homology 2 (SH2) domains of numerous signalling proteins such as PI3-K, Grb2 and SHP2 that are involved in cellular metabolism, mitogenesis, steroidogenesis and gene transcription. Activation of PI3-K, and signalling events downstream of this, couples the insulin signal to glucose uptake, utilization and other aspects of carbohydrate metabolism (Burks et al., 2000; Mathaai et al., 2000). There appears to be tissue selectivity in the activity of PI3-K in the insulin resistance of PCOS. Insulin-stimulated PI3-K activity was observed to be unimpaired in cultured skin fibroblasts from women with PCOS, but IRS-1-associated PI3-K activity was significantly attenuated in skeletal muscle from PCOS patients (Venkatesan et al., 2001). Our data are consistent with the view that PI3-K activity is impaired in granulosa cells from anovulatory women with PCOS.

This study also supports, and extends to granulosa-lutein cells, our previous observations (Willis et al., 1998) that in women with anovPCO, granulosa cells from unstimulated antral follicles are significantly more responsive to LH in terms of progesterone production than those from size-matched follicles of normal and ovPCO. We have previously
hypothesized that hyperinsulinaemia contributes to the mechanism of anovulation seen in women with PCOS by interacting synergically with LH to augment steroidogenesis and induce premature arrest of follicle development (Willis et al., 1998). Further evidence for the association between insulin levels and anovulation is provided by observations that insulin resistance is more severe in women with anovulatory PCOS than in women with a polycystic ovarian morphology, but normal, ovulatory cycles (Conway et al., 1989; Robinson et al., 1993). In addition, studies using insulin-sensitizing drugs such as metformin and troglitizone, which increase insulin sensitivity in PCOS, also appear to enhance spontaneous ovulation and the induction of ovulation, although the mode of action of these drugs is different (Nestler et al., 2002). By demonstrating that insulin resistance in the ovary appears to be confined to the action of insulin on glucose metabolism, the results of the present study provide an explanation for the proposed effect of insulin on steroidogenesis and differentiation of granulosa cells of PCOS in the face of peripheral insulin resistance.

In conclusion, the data presented here show that insulin--but not LH-mediated glucose metabolism is abnormal in anovulatory women with PCOS. However, the response to insulin-stimulated progesterone production is the same in cells from normal and polycystic ovaries. This suggests that there is a divergence in the post-receptor signalling pathways for insulin in the anovulatory polycystic ovary, such that there is a defect in the pathway that regulates glucose metabolism, but not in those regulating ovarian steroidogenesis. This attenuation in the glucose response to insulin could lead to a reduction in the energy supply to the growing follicles, thus contributing to the mechanism of anovulation seen in insulin-resistant women with anovPCOS. In addition, the enhanced response in LH-stimulated progesterone production that we have demonstrated adds further weight to the hypothesis that raised insulin, LH and androgen concentrations are all involved in prematurely advancing granulosa cell differentiation and the arrest of follicle growth (Willis et al., 1998).

**Acknowledgements**

This work was supported by grants from the Medical Research Council, UK and Wellbeing (Royal College of Obstetricians and Gynaecologists), UK.

**References**


Submitted on May 18, 2004; accepted on October 15, 2004