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INSULIN SIGNALLING IN GRANULOSA CELLS

Thesis for the degree of Doctor of Philosophy

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**Declaration**

I, Jalini Joharatnam declare that the following thesis is my own original work and that any published or unpublished work of another person has been duly acknowledged or referenced.
This thesis is dedicated to my parents,

Joe and Gowri Joharatnam

who taught me the value of education, to believe in myself and to always set my sights high

Also, this thesis is dedicated to my husband Praveen who has been a constant source of support and encouragement
Abstract

Polycystic ovary syndrome (PCOS) is characterised by hyperandrogenism and insulin resistance. Granulosa cells in PCOS demonstrate impaired insulin-induced glucose uptake and lactate accumulation, suggesting a post receptor, signalling pathway-specific impairment of insulin action. Gonadotrophins are also important in the regulation of glucose metabolism by granulosa cells.

The first objective of this project was to use KK1 cells an immortalised mouse granulosa cell line, to characterise insulin, androgen and FSH signalling as well as glucose metabolism. Cell lysates were subjected to western immunoblotting for key proteins in the insulin signalling pathways. Glucose metabolism of KK1 cells was also measured. Surprisingly, androgen alone stimulated glucose uptake and lactate production and augmented insulin-induced glucose metabolism. This suggests that the insulin resistance observed in granulosa cells from women with PCOS is not a direct effect of exposure to androgen.

The main part of the thesis was examination of insulin action on human primary ovarian granulosa-lutein (GL) cells to investigate the mechanism of insulin resistance in PCOS. Insulin and FSH signalling in GL cells from women with anovulatory PCOS (anovPCO, n=11) was compared to that in GL cells from ovulatory women with (ovPCO, n=8) and without polycystic ovaries (controls n=12). Primary GL cells were incubated with insulin or FSH and analysed for glucose metabolism and progesterone production. Cell lysates were prepared for identification of signalling pathways, using western
immunoblotting.

The results confirmed selective impairment of glucose metabolism in cells from anovulatory PCOS. No significant impairment of insulin stimulated PI3K signalling was observed. However there was a reduction of p42/44ERK phosphorylation in the ovulatory PCOS group compared to controls. The significance of this finding with respect to impaired glucose metabolism in granulosa cells remains to be determined. We also showed FSH induced glucose metabolism, but without clear evidence of activation of the PI3-kinase pathway.
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Chapter 1

Introduction
1.1 Polycystic ovary syndrome (PCOS)

PCOS is the commonest endocrine disorder of women, affecting 5-10% of the population. It is a heterogeneous syndrome, but classical features include anovulation (irregular menstrual cycles) and symptoms of androgen excess such as hirsutism, acne and alopecia. Biochemically it is common to see high levels of LH with normal FSH levels and high androgen levels. PCOS is also associated with metabolic abnormalities, in particular insulin resistance and hyperinsulinaemia, which carry a higher risk developing type 2 diabetes (1-7).

Controversy surrounds the definition of PCOS. The NIH criteria (1990) identify androgen excess and ovarian dysfunction in the absence of other causes, which may present with symptoms similar to PCOS, no mention is made of ovarian phenotype in this classification. Under the 2003 Rotterdam criteria however two of the following three criteria are required for the diagnosis of PCOS: clinical or biochemical hyperandrogenism, polycystic ovaries on ultrasonography, and menstrual irregularities (8). One of the main aims behind the revised Rotterdam criteria is to highlight and accommodate the increasing evidence that the spectrum of biochemical and clinical features of women with polycystic ovary syndrome is very wide, wider than that allowed by the NIH criteria. This classification has the advantage of highlighting the heterogeneity of the disorder.

One of the main controversies of the Rotterdam criteria is the inclusion of 2 subgroups of patients, those with polycystic ovaries on imaging and irregular periods, and those with polycystic ovaries and hyperandrogenism, women who would not on the NIH criteria be recognised as having PCOS.
However, ‘The Androgen Excess Society’ guidelines (2006) emphasise that androgen excess is the foundation of PCOS (9) and indeed, elevated serum levels of serum testosterone are the most consistent biochemical feature of PCOS.

Insulin resistance is also central to the nature of polycystic ovary syndrome. Insulin sensitivity varies with the PCOS phenotype (10), it is most prevalent in women with anovulation and hyperandrogenism (11). Overall insulin resistance and compensatory high insulin levels affect 65-70% of women with PCOS (12-14). Many studies have shown that both lean and obese women with PCOS have insulin resistance (3). High levels of insulin increase androgen levels, and acts synergistically with gonadotrophins to augment steroidogenesis (15-17). Insulin in combination with LH also prematurely enhances follicle arrest at earlier stages than normal, contributing to anovulation.

1.2 Developmental origin of PCOS and its evolution during childhood

PCOS is thought to be a genetic disorder that is heavily influenced by environmental factors, such as diet (18, 19). The fact that PCOS presents in adolescence, suggests that the origins of the disease occur much earlier than this, most probably in fetal life. Studies done in female Rhesus monkeys which were exposed to high concentrations of testosterone in utero, develop as adults many of the features of women with PCOS, such as high LH levels, ovarian hyperandrogenism, anovulation in relation to increased body weight, and insulin resistance (20, 21). This suggests that the PCOS phenotype could
be a direct result of androgen exposure, which could programme the fetal hypothalamic-pituitary-ovarian axis in utero and may predispose to insulin resistance (figure 1.1).

In the case of the Rhesus monkey model of PCOS, it is important to note that the doses of androgen that were used were high enough to exceed two normally very efficient physiological barriers: firstly, placental aromatase, which converts excess androgen to oestrogens, and secondly, high circulating sex hormone binding globulin (SHBG) which binds testosterone and prevents placental transfer. It is therefore unlikely that, in pregnant women with PCOS, any excess maternal androgen would be able to cross the placenta in significant amounts (22). So “vertical transmission” of PCOS from mother to fetus probably does not occur. It is therefore suggested that the source of excess androgen is the fetal ovary (and/or adrenal) which may either be actively secreting excess androgen in utero or, perhaps more plausibly, be genetically predisposed to produce high ovarian androgen levels at the time of activation of the hypothalamic-pituitary-ovarian axis in infancy or at puberty (23). If the adrenal is contributing to excess androgen in PCOS, the normal burst of androgen secretion at adrenarche may be advanced or enhanced and there is indeed evidence for links between premature adrenarche, premature pubarche and the appearance of PCOS in adolescence (24).
Chapter 1

Figure 1.1: Adapted and revised from Abbott, Dumesic and Franks (23) and Franks McCarthy and Hardy (25). It is proposed that the ovary is genetically predisposed to androgen hypersecretion at the activation of the HPO (hypothalamo-pituitary-ovarian) axis, which occurs transiently in infancy and in a more sustained fashion at puberty. Higher androgen levels programme the HP axis to produce increased LH and amplify the physiological insulin resistance of puberty. High LH and insulin levels act synergistically to increase androgen levels further and may contribute to the mechanism of anovulation (26).

Normal pubertal development is characterized by activation of the hypothalamic-pituitary-ovarian axis and is associated with physiological changes in insulin sensitivity. In girls with a predisposition to PCOS, these changes are exaggerated, for example by higher than normal levels of LH and, significantly, by hyperinsulinemia that is further amplified by obesity.

1.3 Genes and PCOS

The aetiology of PCOS is unknown but heritability studies suggest a strong genetic susceptibility (27). Familial clustering has been noted (19) and also greater concordance of symptoms is seen in monozygotic twins compared to dizygotic twins (28). Studying complex diseases such as PCOS and diabetes is difficult due to their likely genetic heterogeneity (29). However, recently two genes have been highlighted as important.
It is known that common variants in a 47kb region of the first intron of the fat mass and obesity associated gene FTO, influences susceptibility to type 2 diabetes, via a substantial effect on BMI and fat mass (30). Barber et al. showed a significant link between the FTO genotype and PCOS status in a UK case control analysis (31). The association was most evident in obese PCOS patients. The FTO gene predisposes to common obesity and also an altered susceptibility to PCOS, suggesting a mechanistic link between them, which is most likely mediated by fat mass.

Using linkage disequilibrium an association has been found between a dinucleotide repeat marker D19S884, closely linked to the insulin receptor gene on chromosome 19p13.2 and PCOS. This maps to intron 55 of fibrillin 3 (FBN3). Moreover this locus is associated with metabolic features of the syndrome in reproductive age women and their brothers (32). The function of FBN3 is not clear but fibrillins are TGF\(\beta\) binding proteins, and growth factors in the TGF\(\beta\) family have been implicated in early follicle development and theca formation in the ovary (33).

The candidate gene approach is time consuming and has been quite ‘hit and miss’ and genome wide association studies similar to that undertaken for Type II diabetes (34) may be a more successful method of identifying other candidate genes.

1.4 Normal human ovarian cycle

Women are born with multiple primordial follicles, which can stay in an arrested state for up to 50 years. Regular recruitment of primordial follicles to growing follicles begins at puberty. A few follicles recommence growth each
day. A primordial follicle that has commenced growth passes through three stages of development, a primary or pre-antral follicle, secondary or antral follicle and finally a pre-ovulatory follicle. Throughout the cycle this trickle of growing follicles of various stages grow without gonadotrophic support, and do not secrete significant amounts of steroid hormones. Antral follicles are doomed to atresia unless rescued by FSH. At the beginning of the menstrual cycle, roughly 10 small antral follicles undergo a few days of rapid growth until they reach 10mm by the mid follicular phase. One (or occasionally) two of these rescued follicles survives and grows to become the dominant antral follicle and produces high levels of serum oestradiol (35, 36), which suppresses FSH secretion thus reducing blood levels (37). This suppression of FSH does not affect the dominant follicle, as it has plentiful FSH receptors. Evidence from the rat suggests that both oestradiol and FSH play an important part in acquiring LH receptors (38), and only that follicle in which granulosa cells contain the LH receptor can respond to the mid cycle LH surge.

The LH surge stimulates ovulation and the successful ovulatory follicle then converts into the corpus luteum and secretes progesterone and oestradiol until luteolysis 14 days later. LH and FSH levels are at their lowest during this phase of the cycle due to negative feedback from the high oestradiol and progesterone levels (37), and this suppresses further development of small antral follicles (figure 1.2).

After luteal regression the secretion of FSH and LH begin again to rise and further maturation of antral follicles will begin again at this stage.
1.4.1 Androgen and oestrogen production in the follicle

In women, androgen is the main steroid made by thecal cells, and aromatase activity is highest in granulosa cells (39). It is generally assumed that interaction occurs between the two cell types in the follicle with LH stimulating androgen production from the thecal cells, which is then used by the granulosa cells to produce oestrogen, this is known as the two-cell two-gonadotrophin concept (figure 1.3). There is strong evidence that mixing the two cell types in vitro enhances oestrogen synthesis (40) and in vivo the use of testosterone antiserum infusion into the ovarian artery of the sheep ovary bearing the dominant follicle, inhibited oestradiol production by 50% (41). These experiments support the idea that androgen precursors leave the theca cells before being aromatised to oestrogens by the granulosa cells; this is the basis of the two-cell two-gonadotrophin concept. This is thought to be a two
way process however. A reduction of androgen production has been seen from thecal tissue culture after granulosa cell removal (42, 43) and also LH stimulated androgen production from culture rat theca was greater after FSH pre-treatment. Chang et al. have shown that in PCOS patients androgen production is enhanced by intravenous FSH administration which supports a granulosa-theca cell paracrine interaction (44).

Figure 1.3: Two-cell two-gonadotrophin concept

1.5 Abnormal antral follicle development in PCOS

In women with polycystic ovaries, numerous arrested antral follicles are seen with a maximum diameter of 5-10mm (figure 1.4). Despite follicle arrest, granulosa cells from a subset of follicles in anovulatory women with polycystic ovaries hypersecrete oestradiol (45, 46), have increased aromatase activity and progesterone production compared to size matched follicles from normal or polycystic ovaries from ovulatory women (45, 47).
Figure 1.4: Ultrasound appearance of a polycystic ovary (48)

This appears to reflect a state of advanced maturation of medium sized antral follicles. Steroidogenesis by thecal cells is also abnormal with an increase in testosterone and androstenedione (49), this is seen in women with both ovulatory and anovulatory PCOS (50). PCOS is characterised by hypersecretion of LH and FSH levels that are below the normal early follicular phase concentrations. This is likely to be a reflection of the slightly elevated levels of oestradiol (compared to the normal early follicular phase), which lead to suppression of FSH. It may also be partly due to a persistently rapid GnRH pulse frequency of one pulse an hour. This favours pituitary production of LH over FSH, (51-53), which produces the high LH/FSH ratio seen in PCOS (figure 1.5) (54). The elevated LH level stimulates the theca to produce high androgen levels and low FSH levels impair aromatisation of androgen to oestrogen. The addition of FSH in vitro or in vivo increases oestradiol levels and in vivo helps normal follicle development to resume (55).
LH also triggers terminal differentiation and follicle growth arrest in the normal mature preovulatory follicle (56). In normal ovulatory follicles the dominant follicle only responds to LH when it is about 10mm in diameter. In follicles from women with anovulatory PCOS the response to LH occurs inappropriately in a proportion of smaller follicles.

However many patients with PCOS have normal LH levels (57), in these patients elevated serum concentrations of insulin may contribute to follicle arrest. In human granulosa cells of both normal and polycystic ovaries insulin in the absence of gonadotrophins stimulates oestradiol and progesterone production, and has been shown to augment LH induced steroidogenesis in granulosa cells (58). Therefore either high LH levels or amplification of LH action by insulin may cause follicle arrest (figure 1.6) (59).
1.6. **Classic insulin action in target tissues**

The insulin signalling pathway is illustrated in figure 1.7. The insulin receptor (IR) has two extracellular α and two transmembrane β subunits that have protein tyrosine kinase activity. Insulin binding activates an autophosphorylation between the two β subunits, activating its receptor tyrosine kinase activity, which then phosphorylates insulin receptor substrate 1 (IRS1) at several tyrosine kinase residues. Phosphoinositide 3 kinase (PI3K) consists of a regulatory subunit p85 and a catalytic subunit p110, the SH2 domain of the p85 interacts with the phospho-tyrosine residues on the IRS1. Through this interaction PI3K is recruited to the plasma membrane and then converts phosphatidylinositol 4,5 bisphosphate (PIP2) to phosphatidylinositol 3,4,5 trisphosphate (PIP3).

The serine threonine kinase Akt (otherwise known as protein kinase B, PKB) plays a pivotal role in the metabolic actions of insulin. Akt is necessary for insulin action as has been shown by studies utilizing Akt2 knockout mice.
and by ablation of Akt action, using small interfering RNA (siRNA) (61). Akt has a PH domain which binds PIP3 but this interaction alone does not activate it. In order to activate it, phosphorylation at 2 sites, Thr308 and Ser 473 must occur. PDK1 catalyses the phosphorylation and activation of Akt only in the presence of lipid vesicles containing PIP3, which implies Akt activation is PIP3 dependent. Both Akt and PDK1 have PH domains, which bind the PIP3. When Akt binds PIP3 it undergoes a conformational change, exposing the Thr308 binding site to PDK1. The mammalian target of rapamycin (mTOR) is the protein kinase that activates Ser 473. It is usually bound to a regulatory unit called the rapamycin insensitive companion of mTOR (RICTOR), together the mTOR-RICTOR complex is known as mTORC2. The exact mechanism by which insulin activates mTORC2 is unknown.

Glycogen synthase kinase 3 (GSK3) inactivates glycogen synthase by phosphorylating serine residues. Insulin activated Akt therefore inactivates GSK3. Insulin activated Akt/PKB also has functions other than glycogen synthase activation, such as activating GLUT4 translocation (62).
1.6.1 Insulin receptor substrate proteins

There are 4 insulin receptor substrates (IRS) proteins. Although these proteins are highly homologous they appear to provide a complimentary role to each other in insulin/IGF-1 signalling. IRS-1 knockout mice develop insulin resistance in peripheral tissues and impaired glucose tolerance (63, 64). IRS-2 knockout mice exhibit insulin resistance in both peripheral tissues and the liver, and also cause defective growth of islet cells in the pancreas. The combination of multi-factorial insulin resistance and decreased β-cell mass leads to type 2 diabetes (65). The roles of IRS 3 and 4 are less clear in cultured cells.

1.6.2 Glucose uptake; the role of GLUT4

In 1980 it was reported that in rat adipocytes, insulin triggered the movement of a ‘sugar transporter’ from an intracellular store to the cell membrane (66).
This transporter was later characterised as the glucose transporter 4 (GLUT4). Tissues such as the brain have a high glucose need and have glucose transporters that are constitutively located at the cell membrane (GLUT1-3). Mammalian tissues such as muscle and fat, are able to respond to stimuli such as exercise or food absorption, by up-regulating glucose transport by 10-40 fold within minutes using GLUT4.

GLUT4 has been found and extensively studied in insulin responsive tissues such as muscle and adipose tissue. One drawback of using these tissues to resolve the exact positioning and movement of GLUT4, has been that the cytoplasm in these cells is compressed by either myofibrils, or fat droplets, causing poor visualisation. Little work has been done on investigating GLUT4 expression in granulosa cells.

Abnormally low GLUT4 expression has been shown in adipocytes from subjects with PCOS (67) and skeletal muscle from type 2 diabetics (68). GLUT4 expression is mostly restricted to insulin sensitive tissues such as skeletal muscle, cardiac muscle and adipose, however it has been found in the granulosa cells of sheep (69) and mice (70).
1.6.3 MAPK signalling

The activation of the PI3K signalling pathway by insulin stimulates the metabolic functions of insulin, such as glucose uptake and glycogen synthesis. However there is a divergence of the insulin signalling pathway following activation of the insulin receptor. The insulin receptor once activated can bind SHC (a mammalian adaptor protein) and activate the MAPK signalling pathway, which activates certain mitogenic actions of insulin such as cell metabolism, growth, differentiation and steroidogenesis (figure 1.8). Insulin resistance in PCOS appears to be selective affecting metabolic but not mitogenic activity of insulin (71, 72). In insulin resistant states when pancreatic β cells are continuing to compensate, insulin levels rise overcoming insulin resistance and maintaining glucose levels. However the hyperinsulinaemia can lead to an overstimulation of mitogenic actions. This selective insulin resistance may explain how insulin acting through the insulin receptor can stimulate the overproduction of androgen from thecal cells in women with PCOS, despite resistance to the effect of insulin on glucose metabolism (15, 73).
Mitogen activated protein kinase (MAPK) pathways are involved in cell proliferation, growth, differentiation and apoptosis. These functions are closely related to energy levels of the cells and a signal network between ERK and 5’ adenosine monophosphate-activated protein kinase (AMPK) has emerged as a key regulator.

There are five major groups of MAP Kinases, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK and ERK 5. ERK 1 and 2, were cloned in 1991 by Boulton et al. (74). ERK 1 and 2 are very similar and activated by insulin, cytokines, growth factors such as insulin like growth factor 1 (IGF-1) and epidermal growth factor (EGF) acting via receptor tyrosine kinases (RTKs), and hormones acting via the G-protein linked receptors such as LH and FSH. Both ERK 1 and 2 are inactive when not phosphorylated, and require dual phosphorylation for activation at a
threonine and a tyrosine residue. Phosphorylation is likely to result in a conformational change, which allows substrate to bind (75).

Each MAPK pathway contains a three tiered kinase cascade comprising of a MAP kinase kinase kinase (MAPKKK, MAP3K, MEKK or Raf), which phosphorylates a MAP kinase kinase (MAPKK, MAP2K, MEK), which in turn phosphorylates the MAPK. MEK1 activates p44 through phosphorylation of Thr202/Tyr 204 and MEK2 activates p42 through phosphorylation of Thr 185/Tyr 187. Deactivation in vitro occurs via phosphatases.

Insulin acts via the insulin receptor a tyrosine kinase receptor. Subsequent autophosphorylation of the cytoplasmic tails of the receptor on tyrosine leads to tyrosine phosphorylation of SHC. SHC then recruits GRB2-SOS complex (growth factor receptor bound protein 2- Son of Sevenless protein) to the cell membrane. SOS can then catalyse an exchange of the GDP bound to Ras (a GTPase) for a GTP. Ras then recruits serine/threonine kinase Raf to the cell membrane, which becomes activated and phosphorylates MEK.

There is some cross talk between the MAPK and PI3K signalling pathways. It has been shown that activated Ras can interact directly with the catalytic unit of PI3K and cause its activation (76) (77). PI3K can also activate ERK via Rac (a GTPase) and p21-Activated Kinase (PAK). Rac and cell division cycle-42 (CDC42) can synergize with Raf to promote Raf and subsequent ERK activation. It has also been shown that Wortmannin, a PI3K inhibitor can also block the MAP Kinase cascade (78).
A role for MAPK in glucose transport

MAP kinase is capable of phosphorylating an impressive array of target proteins in vitro, many of which have a regulatory role and are known to be phosphorylated in response to insulin. For example, GSK-3 α and β are phosphorylated and inactivated by two protein kinases ribosomal protein S6, the 90kDa S6 (also known as 90rsk or MAPKAP-K1) (79) and 70KDa S6 which lie downstream of MAPK (80, 81).

But this only gives us circumstantial evidence in favour of a role for MAPK in insulin action. Studies ablating MAPK activity using truly specific inhibitors or mRNA targeting have been hampered by the presence of multiple related MAPK isoforms (82).

1.6.4 FOXO proteins

The FOXO proteins are a family of transcription factors, highly conserved from C elegans, and Drosophila to man (83). In the nucleus, FOXO proteins are transcription factors that act to activate or repress certain target genes, by direct binding of the forkhead domain to DNA (84). This is usually in a tissue specific manner. Various deletion studies have highlighted the importance of certain FOXO proteins. FOXO-3a -/- mice exhibit a distinct phenotype of global follicular activation followed by early death of functional ovarian follicles, and therefore premature ovarian failure (85). Complete deletion of FOXO1 in mice is lethal to embryos (86).

FOXO1 expression, in granulosa cells in vivo, is hormonally induced by FSH and oestradiol and down regulated in response to LH during luteinization. In cultured granulosa cells, FSH and IGF-I rapidly phosphorylate FOXO1 via
activation of the PI3K pathway, leading to its exclusion from the nucleus. FOXO1 acts as a co-repressor. Insulin and IGF1 acting via the PI3K pathway, phosphorylate FOXO1 and relieve this inhibition (87). The phosphorylated FOXO1 is inactive, its DNA binding affinity is reduced and it is expelled from the nucleus (88).

In cultured granulosa cells FSH also rapidly down-regulates the expression of the FOXO1 gene. Therefore FSH and LH in vivo act to suppress the functions and expression of FOXO1 (89). Recent studies indicate that FOXO1 is a negative regulator of FSH-mediated proliferation and differentiation (90). Thus, in granulosa cells there appears to be an FSH receptor FOXO1 regulatory loop (91).

1.7 Glucose metabolism

Anaerobic metabolism

Early anaerobic metabolic steps can convert monosaccharides, via pyruvate to acetyl coA. Under anaerobic conditions, pyruvate is converted to lactate by the enzyme lactate dehydrogenase (LDH), and the lactate is transported out of the cell into the circulation. The conversion of pyruvate to lactate, under anaerobic conditions, provides the cell with a mechanism for the oxidation of NADH (produced during glycolysis) to NAD+, which occurs during the LDH, catalyzed reaction. This reduction is required since NAD+ is a necessary substrate for G3PDH (glyceraldehyde-3-phosphate dehydrogenase) an enzyme within the glycolysis pathway (figure 1.9).

As there is no reverse reaction of pyruvate to phosphoenolpyruvate, the pyruvate must first be converted to oxaloacetate, which can then be converted
to glucose by gluconeogenesis.

**Aerobic Metabolism**

Alternatively glucose is converted by glycolysis to pyruvate and then to acetyl CoA, which enters the citric acid, also known as the tricarboxylic acid (TCA) cycle where it can be converted to triglycerides or amino acids. The TCA cycle is a central metabolic pathway that completes the oxidative degradation of fatty acids, amino acids and monosaccharides. The TCA cycle has two main purposes, to increase the cells ATP producing potential, and to provide the cell with a variety of precursors, which can be used to build molecules.

Aerobic glycolysis has the advantage of generating substantially more ATP per mole of glucose oxidized than anaerobic glycolysis, but it is 100 times slower. Anaerobic metabolism is therefore particularly useful when anabolic reactions are not needed, but the generation of as much ATP as possible in the shortest time frame is needed, for example muscle cells during exercise.
Granulosa cell glucose metabolism

Glucose metabolism by granulosa cells is important in providing substrates for the developing oocyte (92). The average glucose concentration of follicular fluid is 3.3mM, compared to a plasma glucose of about 5mM (93). The follicle is a relatively oxygen depleted environment, lactate concentrations in human serum range between 1.5-2mM (94), considerably lower than the average lactate level in follicular fluid of 6.1mM (93), which suggests lactate is produced locally in the follicle and secreted into follicular fluid. This is supported by the fact that gonadotrophins are known to stimulate glucose consumption and lactate production by cumulus cells (95). FSH also induces cumulus cell-enclosed mouse oocyte expression of the glycolytic enzyme hexokinase (96). Follicular fluid levels of pyruvate from women undergoing

Figure 1.9: An overview of carbohydrate metabolism and the TCA cycle.
IVF are in the order of 0.3mM, which is higher than the average plasma pyruvate of 0.08mM (93). Isolated mouse cumulus cells (97) have been shown to produce pyruvate from glucose which may explain this 3-5 fold rise.

Most studies concur that mouse ovarian follicles produce large amounts of lactate during growth and development and prefer a predominantly glycolytic mode of energy production to sustain growth and steroidogenesis. The glycolytic rate is stimulated by gonadotrophins. The amount of lactate produced suggests that ovarian follicles behave like solid tumours, predominantly metabolising glucose via glycolysis (98). This is supported by the observation that when oxidative phosphorylation is prevented using a TCA cycle inhibitor, follicles are still able to undergo normal development and steroidogenesis (99).

1.8 Insulin resistance

1.8.1 Endogenous insulin resistance

In addition to tyrosine phosphorylation both the insulin receptor and IRS proteins undergo serine phosphorylation, which can reduce signalling by attenuating insulin induced tyrosine phosphorylation (100). These inhibitory phosphorylations provide negative feedback to insulin signalling and provide a mechanism for other signalling pathways to have an effect on insulin resistance. Several kinases have been implicated in this process including GSK-3, PKC mTOR and Akt (101).

Insulin receptor tyrosine phosphorylation can also be attenuated by protein tyrosine phosphatases (PTP). One in particular, cytoplasmic phosphatase PTP1B, has been shown to be interesting. Knockout of PTP1B leads to an
increase in tyrosine phosphorylation of insulin receptor and IRS protein in muscle together with improved insulin sensitivity (102).

1.8.2 Causes of insulin resistance

Insulin resistance is characterised by defects at many levels, decreases in receptor concentration and kinase activity, glucose transporter translocation and concentration, and phosphorylation of IRS-1 and PI3K activity (103). MAPK activity is not reduced in Type 2 Diabetes, and it has been suggested that this may provide a mechanism for some of the detrimental effects of chronic high insulin levels on cell growth in vessels (104, 105). Studies in bovine vascular smooth muscle cells have shown a three fold mitogenic response to insulin via the MAPK pathway (106). Activation of MAPK leads to the proliferation and migration of vascular smooth muscle cells contributing to atherosclerosis (107, 108).

Genetic factors can profoundly affect insulin sensitivity. Targeted gene deletions have helped provide some insight into the complexity of these mechanisms. For example although knock outs of the insulin receptor (109), Akt2 (60) and IRS-2 cause diabetes (60, 65, 110), knockout of IRS-1 (63, 64), p85 regulatory subunit of PI3K (111) and GLUT4 (112) do not.

1.8.3 The role of adipose in insulin sensitivity

Fatty Acids and Adipokines

Circulating free fatty acids are elevated in insulin resistance states and are thought to contribute to insulin resistance in diabetes by interfering with glucose uptake, glycogen synthesis and glucose oxidation (113). They are
also thought to be involved in attenuating the response to IRS1 phosphorylation to insulin (114).

The fat cell produces hormones known as adipokines, examples of which include tumour necrosis factor α (TNF-α), leptin and adiponectin. TNF-α has been shown to produce serine phosphorylation of IRS-1 (100) and inhibition of TNF-α significantly improves insulin resistance (115). Severe leptin deficiency has been characterised by insulin resistance states. Genome wide scanning has shown a susceptibility locus for type 2 diabetes at chromosome 3q27 in the region of the adiponectin gene. Insulin resistance in lipoatrophic mice was completely reversed by replacement of adiponectin together with leptin. Treatment with either leptin or adiponectin alone produced only partial reversal (116).

1.8.4 Insulin resistance in PCOS

It has become clear over the last 25 years that polycystic ovary syndrome is an important metabolic disorder. In 1980 Burghen et al. reported that obese women with PCOS had significantly elevated basal and post glucose load insulin levels compared to weight matched control women, suggesting women with PCOS were resistant to insulin (117). Insulin mediated glucose uptake is decreased by 35-40% compared to age and weight matched control women (3, 118). Subsequent studies determined that high insulin levels are characteristic of women with PCOS, independent of obesity (10). Many studies have shown that both lean and obese women with PCOS have insulin resistance (3).
Insulin resistance is an inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilisation as much as it does in the normal population. The main consequences are a reduced ability of insulin to suppress hepatic gluconeogenesis and stimulate peripheral glucose uptake. It would be expected that circulating glucose levels would rise as a consequence, however provided β cell function of the pancreas is adequate, insulin secretion increases to overcome this insulin resistance and glucose levels are normalized. Compensatory high insulin levels are therefore the hallmark of insulin resistance. When this compensatory mechanism fails type 2 diabetes occurs. However glucose stimulated insulin release is also inappropriately low in women with PCOS suggesting there are further defects in pancreatic β cell function (118). Women with PCOS have a 3-4 times greater risk of developing type 2 diabetes (119, 120).

Although common insulin resistance it is not a universal feature of all women with PCOS. Interestingly insulin resistance is most prevalent in women with PCOS who have both androgen excess and anovulation (i.e NIH criteria), independent of polycystic ovaries. Whereas weight matched women with ovulatory cycles, who are equally hyperandrogenaemic have normal insulin sensitivity and serum insulin concentrations (figure 1.10).
Rotterdam phenotypes of polycystic ovaries and hyperandrogenism with ovulatory cycles or polycystic ovaries with normal androgen levels and anovulation have milder metabolic dysfunction. Whereas women with polycystic ovary phenotype and regular cycles are metabolically normal although may have subtle hormonal abnormalities (12, 121).

Insulin resistance has been implicated in the mechanism of anovulation seen in PCOS patients. Insulin in combination with LH prematurely enhances follicle arrest at earlier stages than normal, contributing to anovulation. Therapies that lower insulin levels such as insulin sensitising drugs and weight loss significantly improve menstrual cyclicity, fertility, and hyperandrogenism, in both lean and obese patients, which suggests insulin
resistance plays an important role in the aetiology of anovulation in PCOS (122) (123).

Paradoxically, insulin greatly augments the action of LH on steroidogenesis (58), thus implying that some actions of insulin on the ovary in PCOS (notably on steroidogenesis and growth/differentiation pathways) are maintained in the face of peripheral insulin resistance. This could result in an enhancement of oestradiol and progesterone production from granulosa cells, causing the suppression of follicle stimulating hormone (FSH) and premature arrest of follicular growth (59). However as indicated below, the metabolic actions of insulin in granulosa cells appear to be impaired.

1.8.5 Mechanism of insulin resistance in PCOS

The exact mechanism of reduced insulin sensitivity in PCOS is unknown. Potential areas of disturbance in the insulin signalling pathway are highlighted below in Table 1, adapted from Diamanti-Kandarakis et al. 2006 (124). This table highlights the variability of defects found in different tissues and also the many areas where information is unknown.
Abnormalities of the insulin receptor itself are rare causes of PCOS and the majority of evidence points to a post-receptor defect in insulin signalling pathways (3, 7, 127). It is likely that the signalling defect in insulin resistance seen in PCOS is located proximally in the pathway, involving serine phosphorylation of the insulin receptor (IR) and/or insulin receptor substrates 1 and 2 (IRS1/2) (3, 127). Isolated skin fibroblasts of about 50% of women with
PCOS who had insulin resistance documented by euglycaemic clamp studies, have constitutively active serine phosphorylation of the insulin receptor that inhibits insulin-stimulated tyrosine phosphorylation and therefore normal signalling (130).

In skeletal muscle cultures from obese women with PCOS, IRS-1 protein abundance was found to be significantly increased, associated with decreased activity of PI3K (127). Phosphorylation of IRS-1 Ser312 (equivalent to Ser307 in rat) was increased in PCOS, but despite these defects, cultured myotubules showed normal insulin responsiveness. No differences in IR tyrosine phosphorylation were seen between women with PCOS and controls. Thus, in contrast to skin fibroblasts, the defects in skeletal muscle seen in vivo are not reproduced in culture, suggesting that the in vivo environment is the major determinant of muscle insulin resistance in PCOS. However certain defects in insulin signalling persist (127). Serine phosphorylation of IRS1 inhibits its tyrosine phosphorylation and therefore its ability to recruit PI3K (135). Another feature of insulin target tissues in women with PCOS is that expression of the insulin-dependent glucose transporter GLUT4 is abnormally low. This has been shown in adipocytes from subjects with PCOS (67) as well as in skeletal muscle from type 2 diabetics (68).

Little work has been done looking into insulin action in granulosa cells. Erickson et al. have shown however that granulosa cells from women with and without PCOS show no differences in oestrogen and progesterone production in response to insulin, suggesting that granulosa cells remain insulin responsive despite peripheral insulin resistance (136). Franks et al. have confirmed the preserved steroidogenic response to insulin, but have also
shown paradoxically a resistance to insulin mediated glucose metabolism in granulosa-lutein cells from women with anovulatory PCOS (71) (figure 1.11). The preservation of steroidogenesis and the impairment of glucose metabolism suggests that there is a post-receptor insulin signalling abnormality affecting glucose uptake and metabolism (71).

![Figure 1.11: Lactate production and progesterone production by cultured granulosa-lutein cells after 48h of incubation in response to insulin (1-1000ng/ml) in women with normal ovaries (open squares), with ovulatory PCO (open diamonds) and with anovulatory PCO (filled circles). Adapted from Rice et al. 2005 (71)](image)

Experimental data from cultured granulosa cells do not support the idea that signalling pathways such as PI3K and MAPK control the steroidogenic activity of insulin as in other insulin target tissues (137, 138). The mechanism of insulin induced steroidogenesis has been attributed to its direct effect on components of the steroidogenic pathway as described in section 1.10.

### 1.9 Gonadotrophin action on glucose metabolism in granulosa cells

Gonadotrophins are also thought to be involved in energy metabolism during follicle development. Hillier et al. showed granulosa cells respond to gondotrophins by increasing lactate production (95). Gonadotrophins are also
believed to enhance the activity of the p38 MAPK (139), ERK (140), and PI3K (141) pathways, the latter being involved in metabolism and uptake (70, 142). PKA selective inhibitors, inhibit the activation of these signalling pathways and target genes, suggesting a PKA dependent mechanism of PI3K and ERK activation (140, 143).

FSH has been shown to have important effects on glucose metabolism. FSH significantly increased oocyte maturation and produced a 2-3 fold increase in glucose uptake and lactate production by cumulus oocyte complexes in which the enclosed oocyte completed maturation (70). In this study, the PI3-kinase inhibitor LY294002 inhibited FSH stimulated glucose uptake, and GLUT4 glucose transporter was found in granulosa cells suggesting that FSH increased glucose uptake by PI3-kinase-mediated translocation of GLUT4 to the granulosa cell membrane (figure 1.12) (70).

Figure 1.12: Insulin and gonadotrophin action on PI3K and MAPK signalling (K Hardy)
Rice et al. used granulosa-lutein cells from women with anovulatory PCOS, ovulatory PCOS, and a control group of women and looked into the effects of insulin (1-1000ng/ml) and LH (1-5ng/ml) on carbohydrate metabolism and progesterone production. Cells from the anovulatory PCO subjects were significantly less responsive to insulin in lactate production than those from both of the other two groups. LH addition however resulted in a dose-dependent increase in glucose uptake, lactate and progesterone production in each group (figure 1.13) (71).

Figure 1.13: Lactate and progesterone production by cultured granulosa-lutein cells after 48h of incubation in response to LH (1-5ng/ml) in women with normal ovaries (open squares), with ovulatory PCO (open diamonds) and with anovulatory PCO (filled circles) (71). LH-stimulated progesterone production (P=0.012, ANOVA b) Adapted from Rice et al. 2005 (71)

1.9.1 Protein Kinase A Signalling

The response of granulosa cells to LH and FSH is mainly mediated by cyclic adenosine monophosphate/ protein kinase A (cAMP/PKA) signalling (figure 1.14). LH binds the LH receptor and FSH binds the FSH receptor both of which are transmembrane receptors. On binding the gonadotrophins both
receptors stimulate the Gs protein (144). GDP bound to the αGs subunit is exchanged for GTP and the αGs-GTP subunit breaks off and binds with adenylate cyclase. This generates cAMP, which then binds the regulatory units of PKA. The complex then dissociates into 2 catalytic subunits and a regulatory subunit. The catalytic subunit then phosphorylates cAMP response binding proteins (CREM and CREB), which bind upstream DNA regulatory elements called cAMP response elements. Genes such as those encoding the LH receptor and P450 aromatase are regulated by this method.

Figure 1.14: Illustration of FSH and LH signalling via the PKA pathway

1.10 Androgen production in normal women and in PCOS

The major androgens in women are dehydroepiandrosterone sulphate (DHEAS) a unique product of the adrenal zona reticularis (AZR); dehydroepiandrosterone (DHEA), androstenedione (A) and testosterone (T) which are all secreted by both the adrenal zona reticularis and ovarian thecal
cells and finally dihydrotestosterone (DHT) which is primarily a product of peripheral testosterone production. Androgen synthesis occurs in both the adrenal and ovary. The first three hormones described above are considered as pro-androgens, requiring conversion to testosterone in order to exert an effect. The regulation of androgen secretion requires ACTH action on the adrenal gland, and LH action on the ovary. The liver, adipose and skin have 3βHSD and 17βHSD, together with aromatase, which allow conversion of androgen to oestrogen (figure 1.15). Androgen biosynthesis is modulated by two critical P450 enzymes, P450 side chain cleavage (P450scc) which catalyses cholesterol side chain cleavage, and P450c17, which catalyzes 17 hydroxylation and 17-20 bond cleavage necessary to make DHEA from pregnenolone and androstenedione from progesterone. The other two very important enzymes are 3β hydroxysteroid dehydrogenase (3βHSD) which catalyses pregnenolone to progesterone and 17β hydroxysteroid dehydrogenase (17βHSD) which catalyses androstenedione to testosterone (145).
The fundamental abnormality in women with PCOS is the overproduction of androgen by the ovary, particularly testosterone by the thecal cells. These cells over express various proteins including LH receptors, insulin receptors, steroidogenic acute regulatory protein (StAR), P450scc and 3βHSD, and P450c17; all these molecules are involved in steroidogenesis (figure 1.15).

1.10.1 Effect of gonadotrophins and insulin on androgen production

The basis for overproduction has been attributed to constitutive increase in theca cell responsiveness to gonadotrophins, in association with the higher pituitary LH levels often seen in women with PCOS. Hyperandrogenaemic women with PCOS have been shown to have excess 17-OHP production in response to GnRH agonist compared to women without PCOS suggesting a
general increase in activity of the androgen biosynthetic pathway (146). This is supported by in vitro work showing cultured thecal cells from PCOS patients produced far greater androgen levels both basally and when exposed to LH, than theca from normal women (50).

1.10.2 Effect of insulin on androgen production

Insulin has a critical role in regulating androgen levels (figure 1.1). Insulin acts to increase androgen levels directly and indirectly. Insulin has been shown to act directly via its’ own receptor at physiological concentrations in cultured thecal cells from women with PCOS to increase androgen production (15). So insulin alone can increase androstenedione production, but also importantly insulin and LH together can increase androgen biosynthesis further (16). In women with PCOS, high levels of LH and insulin act synergistically to raise androgen levels (17).

Insulin can also act indirectly to increase androgen levels by reduction of hepatic production of sex hormone binding globulin (SHBG) levels, thereby raising free androgen levels (147). The effects of insulin on androgen are summarised in Table 2 adapted from Diamanti-Kandarakis et al. 2006.


<table>
<thead>
<tr>
<th>Effects</th>
<th>Tissue</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑Amplitude of LH secretion</td>
<td>Pituitary</td>
<td>(148, 149)</td>
</tr>
<tr>
<td>↓SHBG secretion</td>
<td>Liver</td>
<td>(150)</td>
</tr>
<tr>
<td>↓IGFBP-1</td>
<td>Liver</td>
<td>(151, 152)</td>
</tr>
<tr>
<td>↑Testosterone production</td>
<td>Theca cells</td>
<td>(153)</td>
</tr>
<tr>
<td>↑17α hydroxylase</td>
<td>Theca cells</td>
<td>(16, 153, 154)</td>
</tr>
<tr>
<td>↑3β-HSD</td>
<td>Theca and/or granulosa cells</td>
<td>(155, 156)</td>
</tr>
<tr>
<td>↑Progesterone production</td>
<td>Theca and/or granulosa cells</td>
<td>(157)</td>
</tr>
<tr>
<td>↑P450 activity</td>
<td>Granulosa cells</td>
<td>(158, 159)</td>
</tr>
<tr>
<td>↑Oestradiol production</td>
<td>Granulosa cells</td>
<td>(157)</td>
</tr>
<tr>
<td>↑cAMP and or StAR biosynthesis</td>
<td>Granulosa cells</td>
<td>(160)</td>
</tr>
<tr>
<td>↑LDL receptor</td>
<td>Granulosa cells</td>
<td>(161)</td>
</tr>
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</table>

*Table 2*: Effects of insulin on hyperandrogenaemia in PCOS, upward and downward arrows indicate increased and decreased levels respectively. Adapted from Diamanti-Kandarakis et al. 2006 (124).

### 1.10.3 Non-ovarian sources of excess androgen production

Enzyme dysregulation in the adrenal, ovary, liver and peripheral tissues seem to contribute to hyperandrogenaemia in PCOS. Ovarian thecal cells from PCOS ovaries in vitro demonstrate persistent overexpression of enzymes in the steridogenic pathway, with resulting excess androgen production (162).

In 25% of adults with PCOS there is increased adrenal responsiveness to ACTH, with an accelerated production of DHEA-S and androstenedione (163, 164). This could be due to increased P450c17α activity, which is the rate-limiting enzyme in androgen production in both the adrenal gland and the ovary. In addition androgen action may be enhanced by 5α reductase activity.
in peripheral tissue such as adipose tissue and the liver, which will increase
the production of dihydrotestosterone (DHT) from testosterone (T) (165)
(figure 1.15). DHT has a higher affinity than testosterone for the androgen
receptor and can not be converted to oestradiol.

1.11 Androgen action

1.11.1 Classical androgen receptor signalling

Knowledge of the mode of action of steroid receptors has been extended in
recent years. In addition to classical nuclear action, acting as transcription
factors, they can also regulate cell signalling phosphorylation and exert
actions that are initiated at the cell membrane, and often induce rapid
responses. During classical nuclear action, T normally passively diffuses
across the cell membrane where it can be converted to DHT by the enzyme
5α-reductase. Both DHT and T are able to bind the androgen receptor (AR),
which is found in its unbound state in the cytoplasm attached to heat shock
proteins and cytoskeletal proteins. On binding, a conformational change
occurs which dissociates these proteins and allows binding to co-regulator
proteins, such as importin-α. This promotes AR stability and thereafter nuclear
translocation and activity (166) (figure 1.16).
Figure 1.16: Illustration of the classical androgen receptor (AR) cycle and the non-genomic effects of androgen; NTD (N-terminal transactivation domain), DBD (DNA-binding domain). (1) Conversion of testosterone to DHT via 5α-reductase; (2) DHT moves into the cytoplasm and binds AR; (3) Ligand binding to AR induces AR conformational change, proteins such as heat shock proteins (HSPs) dissociate and others such as importin-α and androgen receptor-associated protein-70 (ARA70) are recruited to help stabilise AR and promote AR nuclear translocation; (4) AR dimerises in the nucleus. Other co-activators bind to AR in the nucleus, the AR-DBD then facilitates nucleic acid binding at androgen response elements (ARE) which promote recruitment of co-activators resulting in chromatin remodeling, and subsequent gene transcription (not shown) (5) Non-ligand-bound AR is shuttled back to the cytoplasm and re-cycled. (6) DHT diffuses through the cytoplasmic membrane and interacts directly with AR where it can exert a ‘non-genomic effect’ with kinases. Adapted from Bennett et al, 2009 (166) and from Gronemeyer et al. 2004 (167).

1.11.2 Non classical androgen receptor signalling

It is only recently that non-classical or non-genomic, rapid (seconds to minutes), membrane initiated testosterone actions have received attention, and has now been seen in reproductive tissues (sertoli cells, oocytes), immune tissues, cardiovascular system and musculoskeletal system. There is evidence for both cytoplasmic AR and novel, membrane bound AR in this non classical action (168). The AR has been shown to interact directly with the
p85 regulatory subunit of PI3K in mouse vas deferens and stimulate Akt phosphorylation, this was then abolished using the AR antagonist bicalutamide (169). Early suggestions were that the rapid effects of testosterone were via aromatisation to oestrogen, and oestrogen receptor (ER) signalling. Compelling evidence for the rapid effects of oestrogen on PI3K and MAPK signalling now exist (170, 171), however this has proven not to be the case using aromatase inhibition and ER inhibition.

1.11.3 Membrane bound androgen receptor

It is now been demonstrated that a proportion of ‘classical’ ARs in certain cells such as sertoli cells and also breast cancer cells localise to the plasma membrane. Treatment with testosterone conjugated to bovine serum albumin (T-BSA), a membrane impermeable analogue, mimics the actions of testosterone and exerts specific effects in both sertoli cells and breast cancer cells, indicating plasma membrane action (172, 173). After testosterone stimulation AR is thought to associate with and activate tyrosine kinase, Src kinase which subsequently activates the MAPK pathway via the EGF receptor (figure 1.17) (168).

It is possible that the AR may also activate Akt via the EGFR. Recently Hammes and Levin introduced the theory of signalling complexes to explain steroid/growth factor interactions. This accepts the co-existence at the plasma membrane of androgen receptor, oestrogen receptor, G coupled protein, growth factor receptors (EGFR, IGFR), and tyrosine kinases (src/ras) and linker proteins such as MNAR (modulator of genomic activity of ER). AR may utilise MNAR as a scaffold in its interactions with growth factors. The close proximity of these molecules in a strict spatially defined area promotes
protein-protein interactions, which may then activate signalling pathways such as PI3K and MAPK (174).

Figure 1.17: Classical ARs activate EGFR in response to ligand by signalling via src and MNAR. (PRB=progesterone receptor B, MNAR=modulator of genomic activity of ER). Adapted from Hammes et al. 2007 (174).

Another possible mechanism of Akt activation by androgen could be via lipid rafts. In LnCaP cells androgen has been seen to bind to membrane bound androgen receptor (mAR) which then interacts with and activates Akt via lipid raft membranes, independently of PI3K (175) (figure 1.18).

PI3K activation and Akt phosphorylation by insulin like growth factor 1 (IGF-1) is also involved in both AR serine phosphorylation and translocation into the nucleus of skeletal muscle cells, which leads to an increase in AR target gene expression (skeletal α-actin) (176).
The role of androgen in male reproduction and prostate cancer has been well defined and many of the studies described above have been in the male reproductive system. However little is known about the direct involvement of androgens and the AR in females. Recently a global androgen receptor knock out (ARKO) mouse was created and mature females were found to have premature ovarian failure (177). Further to this granulosa cell (GC) specific and oocyte specific ARKO mice have now been created. Granulosa cell specific ARKO mice were shown to have atretic follicles, premature ovarian failure and were sub-fertile whereas oocyte specific ARKO had normal fertility and ovarian morphology. This data suggests that the AR in granulosa cells
promotes preantral follicle growth and prevents follicle atresia, and is therefore essential for normal fertility (178).

1.11.4 Effect of androgen on insulin sensitivity

Excess androgen production is a pathophysiological feature of PCOS and exposure to exogenous testosterone in vivo has been associated with development of insulin resistance in rats and human females (179-181). However the mechanism by which hyperandrogenism reduces insulin sensitivity is unknown. In adolescents with PCOS, the risk of developing the metabolic syndrome increases as bioavailable testosterone levels rise, and this phenomenon is independent of obesity (182).

1.11.5 Androgens and insulin resistance

It is thought that high androgen levels and insulin resistance are linked. Insulin resistance is most prevalent in women with PCOS who have both androgen excess and anovulation (figure 1.10). Clinically administration of testosterone to female patients undergoing transformation to the male gender has been shown to induce insulin resistance (183). Administration of anabolic steroids such as methandrostenolone, which acts via the androgen receptor, have also been shown to induce insulin resistance in young athletes (184). Other evidence supporting the adverse effect of sex hormones on insulin sensitivity, comes from children reaching puberty, who have been found using euglycaemic clamp studies to have a reduction in insulin stimulated glucose metabolism (185).

In vitro, intravenous testosterone administration in rats has been shown to produce a polycystic ovarian phenotype, associated with low/normal glucose
and high insulin blood levels (186). Also male rhesus monkeys that have been pre-natally androgenised, have been shown to have diminished insulin sensitivity (187). Corbould et al. investigated the action of androgen on pre-adipocytes from healthy women treated with testosterone or anti-androgens for 48h. Maximal insulin stimulated glucose uptake (insulin 10nM) was impaired in the cells treated with testosterone 10nmol/L and 100nmol/L. This defect was attenuated by co-incubation with anti-androgens. However, testosterone treatment did not alter phosphorylation of IRS-1, AKT or MAPK (188).

1.11.6 Insulin signalling activates androgen signalling

There is also some evidence of a link between insulin and androgen signalling. Liganded AR recruits a protein known as forkhead transcription factor (FOXO-1/FKHR) to the AR promoter area where it interferes with AR-DNA interactions. FOXO1 acts as a co-repressor of AR. Insulin and insulin like growth factor 1 (IGF1) abolish this occupancy, and relieve this inhibition (87). PI3K signalling which is activated by both insulin and IGF1 phosphorylates FOXO1. The phosphorylated FOXO1 is inactive and expelled from the nucleus (88).
1.12 Aim

The overall aim of this project was to investigate the mechanism of insulin resistance in polycystic ovary syndrome in granulosa lutein cells. KK1 cells, an immortalised luteinizing mouse granulosa tumour cell line, expressing well characterised gonadotrophin receptors and steroidogenic enzymes were used initially to investigate the effects of androgen and its’ interaction with insulin on glucose metabolism and PI3K and MAPK signalling (189). Investigating insulin and androgen action in granulosa cells provided a good model for comparison of insulin action in women with PCOS.

FSH has been shown to have important effects on glucose metabolism. FSH significantly increased oocyte maturation and produced a 2-3 fold increase in glucose uptake and lactate production by cumulus oocyte complexes in which the enclosed oocyte completed maturation (70). Gonadotrophins are thought to be involved in energy metabolism during follicle development. Hillier et al. showed granulosa cells respond to gonadotrophins by increasing lactate production (95). The signalling pathways involved in insulin action, androgen action and insulin/androgen interaction in mouse granulosa cells were then compared with those mediating FSH stimulated glucose metabolism and steroidogenesis in KK1 cells.

Insulin and FSH action in human primary granulosa lutein cells from women with normal ovaries (control), those with endogenous androgen excess i.e women with polycystic ovary syndrome who continue to ovulate (ovPCO) and those who have irregular or anovulation (anov PCO) was then compared. The time and dose dependent effects of insulin and FSH action on glucose uptake, lactate accumulation and progesterone production were characterised. Insulin
and FSH action on key phospho-proteins in the PI3K and MAPK signalling pathways was also assessed.

Several studies have shown an effect of FSH on steroidogenesis in human primary granulosa lutein cells taken from normal women undergoing IVF (190-193). However this is the first study in primary human granulosa lutein cells to look at gonadotrophin effects on downstream signalling pathways involved in glucose metabolism. Previous work in luteinized GL cells has looked at the effect of LH but not of FSH.
Chapter 2

Effects of insulin and androgen on glucose metabolic pathways in an immortalised mouse granulosa cell line (KK1)
2.1 Introduction

Polycystic ovary syndrome (PCOS) is characterised by hyperandrogenism and insulin resistance. Insulin resistance is central to the nature of polycystic ovary syndrome and varies with the PCOS phenotype (10), it is most prevalent in women with anovulation and hyperandrogenism (11). Patients with PCOS have reduced insulin sensitivity and a 3-7 times greater risk of developing Type II Diabetes (119, 120). High levels of insulin increase androgen levels, and acts synergistically with gonadotrophins to augment steroidogenesis (15-17). Insulin in combination with LH also prematurely enhances follicle arrest at earlier stages than normal, contributing to anovulation.

Little is known about the mechanism of insulin resistance in PCOS particularly in relation to action in the ovary. Given that high androgen levels and insulin resistance are often found together in PCOS, we decided to assess the impact of androgen on insulin stimulated glucose metabolism and also its effect on insulin signalling. This has not been previously studied in granulosa cells. In previous studies, we demonstrated that the effect of insulin on glucose metabolism in granulosa-lutein cells from women with anovulatory PCOS is attenuated while its steroidogenic response is preserved (71). Glucose metabolism is needed for normal oocyte maturation and function (70) and this defect may contribute to infertility seen in PCOS patients. The preservation of steroidogenesis and the impairment of glucose metabolism suggests that there is a post-receptor insulin signalling abnormality affecting glucose uptake and metabolism (71).
Excess androgen production is a pathophysiological feature of PCOS and exposure to exogenous testosterone in vivo has been associated with development of insulin resistance in rats and human females (179-181). However the mechanism by which hyperandrogenism reduces insulin sensitivity is unknown. In adolescents with PCOS, the risk of developing the metabolic syndrome increases as bioavailable testosterone levels rise, and this phenomenon is independent of obesity (182).

The fundamental abnormality in women with PCOS is the overproduction of androgen by the ovary, particularly testosterone by the thecal cells. These cells over express various proteins including LH receptors, insulin receptors, steroidogenic acute regulatory protein (StAR), P450 side chain cleavage (P450scC) and 3-β-hydroxysteroid dehydrogenase (HSD), cytochrome P450c17 and all these molecules are involved in steroidogenesis (figure 1.15). Enzyme dysregulation in the adrenal, ovary, liver and peripheral tissues contribute to hyperandrogenaemia in PCOS as described in section 1.10.3.

Insulin also has a critical role in regulating androgen levels. Insulin acts to increase androgen levels directly and indirectly. The effects of insulin on androgen are summarised in table 2, section 1.10.2 (124).

It is thought that high androgen levels and insulin resistance are linked. Insulin resistance is most prevalent in women with PCOS who have both androgen excess and anovulation (figure 1.10). Clinically, administration of testosterone to female patients undergoing transformation to the male gender has been shown to induce insulin resistance (183). Administration of anabolic steroids such as methandrostenolone, which acts via the androgen receptor, have also been shown to induce insulin resistance in young athletes (184). Other
evidence supporting the effect of sex hormones on insulin sensitivity, comes from children reaching puberty, who have been found using euglycaemic clamp studies to have a reduction in insulin stimulated glucose metabolism (185).

In vitro, testosterone injection into rats has been shown to produce a polycystic ovarian phenotype, associated with a low/normal glucose and high insulin blood levels (186). Also male rhesus monkeys that have been prenatally androgenised, have been shown to have diminished insulin sensitivity (187). Corbould et al. investigated the action of androgen on pre-adipocytes from healthy women treated with testosterone or anti-androgens for 48h. Maximal insulin stimulated glucose uptake (insulin 10nM) was impaired in the cells treated with testosterone 10nmol/L and 100nmol/L. This defect was attenuated by co-incubation with anti-androgens. However, testosterone treatment did not alter phosphorylation of IRS-1, Akt or MAPK (188).

In addition to classical nuclear action of steroid receptors, acting as transcription factors, androgen receptors can also regulate cell signalling phosphorylation and exert actions that are initiated at the cell membrane, and are often rapid responses (non genomic) (figure 1.16 and 1.17). This is described in greater detail in section 1.11.
Chapter 2

2.2 Aims

The objective of this study was to explore the effects of androgen and its interaction with insulin on glucose metabolism and phosphoinositide 3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling in granulosa cells. KK1 cells are an immortalised luteinizing mouse granulosa tumour cell line, expressing well characterised gonadotrophin receptors and steroidogenic enzymes (189). Investigating insulin and androgen action in granulosa cells provides a good model for comparison of insulin action in women with PCOS.

Specific Aims were:

1. to establish a mouse granulosa cell line to examine the effects of insulin on glucose uptake and metabolism
2. to explore the effect of insulin on the glucose transporter GLUT4
3. to define the signalling pathways involved and to investigate the effect of androgen, in vitro, on insulin mediated glucose metabolism
4. to explore the effect of androgen alone on signalling pathways or glucose metabolism
5. to compare the signalling pathways involved in insulin action, androgen action and insulin/androgen interaction in mouse granulosa cells with those mediating gonadotrophin stimulated glucose metabolism and steroidogenesis.
2.3 **Materials and Methods**

2.3.1 **KK1 cell line**

KK1 cells are an immortalised luteinized mouse granulosa tumour cell line, expressing well characterised gonadotrophin receptors and steroidogenic enzymes (189). We have used these cells to explore the actions of insulin and androgens on Akt, phosphorylated Akt, as well as glucose uptake and metabolism in KK1 cells.

The KK1 cell line (kindly donated by Prof Ilpo Huhtaniemi) was established from transgenic mice. This was achieved by introducing a gene construct containing a 6 kilobase fragment of mouse inhibin α subunit promoter with simian virus 40 T-antigens into one cell stage embryos. Mouse inhibin α-subunit promoter, is a tissue specific promoter, therefore producing transgenic mice expressing the SV40 T-antigen driven by the mouse inhibin α-subunit will allow only specific cell types to undergo transformation. This resulted in the development of tumorous ovaries in female mice. Permanent ovarian cell lines were established by growing cells from explant cultures of these ovaries and then allowing them to grow in monolayers in culture. Subculture 28 gave the strongest cAMP and steroidogenic responses so became further characterised to become the KK1 cell line. This cell line is a good model for granulosa cells as they use the cAMP/ adenylate pathway to produce progesterone.

2.3.2 **Reviving KK1 cells from frozen stock**

Cells were revived from frozen cell stocks. The vial was warmed in the hand to 37°C and added to 5mls DMEM-F12 with phenol red (Invitrogen 31330-
supplemented with 1% penicillin and streptomycin (Sigma P0781) and 10% heat inactivated foetal bovine serum (FBS) (Invitrogen 10108-165) in a 15ml centrifuge tube. This tube was then centrifuged at 200g for 5 minutes using a Centra CL3 (Thermo IEC) centrifuge. The supernatant was discarded leaving a pellet of cells. This step is to ensure the removal of dimethylsulfoxide (DMSO) which was used in the freezing media to prevent the formation of ice crystals which otherwise lyse cells during thawing. 5mls of serum-supplemented medium was then added to the KK1 cell pellet and the centrifuge tube was then vortexed. Following this the cells were added to 75cm tissue culture flasks (TPP), and topped up with 20mls of serum supplemented media. The passage number of the cells and the date was noted and recorded on the flasks.

2.3.3 Cell culture and splitting cells

All culture dishes (Nunc, VWR) and flasks (TPP, Helena Biosciences) were suitable for tissue culture and all serological plugged pipettes were purchased from Costar. All experiments were carried out in a Class II flow cabinet and cells were grown at 37°C in 5% CO₂. KK1 cells were grown in Dulbeco’s Modified Medium (DMEM-F12) supplemented with 10% heat inactivated Foetal Calf Serum (FCS), 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 μM glutamine (Invitrogen, Gibco). Cells were passaged thrice weekly by incubating with 5 ml of 0.25% trypsin/EDTA mix (T3924, Sigma) for 3 minutes. The cells were re-suspended in 5 ml of DMEM-F12 supplemented with 10% heat inactivated FCS, 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 μM glutamine (Invitrogen, Gibco) and plated to the required density.
2.3.4 Freezing KK1 cells after culture

Cells were grown in T75 flasks until confluent. Adherent cells were incubated with 5 ml of 0.25% trypsin. The trypsin was then inactivated; by adding 5 ml of DMEM-F12 with 10% heat inactivated fetal bovine serum (FBS). The cells were spun down in a centrifuge tube and resuspended in freezing mixture (standard media + 10% DMSO + 10% FBS). Cells were then aliquoted into special freezing vials and transferred to a Mr Frosty (freezing container providing 1°C/min cooling rate required for successful cell cryopreservation). This was then placed in the -80°C freezer.

2.3.5 Cell counting

Cells were counted using disposable haemocytometers (Hycor Biomed) using a light microscope (Olympus CK2). The values were converted into cell density based on the volume of the counting chamber. The experiments were repeated 2-3 times to overcome the fact that cell culture experiments can produce variable results on different occasions. For each experiment the KK1 cells were plated in triplicate.

2.3.6 KK1 cell culture, protein isolation and expression

For protein expression experiments, cells were cultured in 6 well plates at a density of $1.4 \times 10^6$ cells/well, in 3mls of serum-supplemented medium 199. For glucose uptake and metabolism, cells were plated in 96 well plates at a density of $2 \times 10^4$ viable cells/well in 200µl of serum-supplemented medium 199. For each experiment the KK1 cells were plated in triplicate. To those KK1 cells being exposed to androgen, Dihydrotestosterone (DHT) (Sigma) was added at this stage. The DHT was dissolved in ethanol (appendix 3), and
the ethanol was tested to ensure it had no effects at the concentrations used. After 24 hours incubation at 37°C in 5% CO₂, the medium was removed and cells washed thoroughly with PBS. They were then incubated in serum free DMEM-F12 +/- 10nM DHT (appendix 3) for 24 hours. Insulin (dissolved in acidified H₂O) was added for various amounts of time at various doses (appendix 3). Cells were lysed in 100 µl ice cold RIPA buffer directly on the plate (appendix 2), with 10 µl protease inhibitors (appendix 2), 10 µl aprotinin (MP Biochemicals) and 10 µl sodium orthovanadate. Using a cell scraper, the lysates were transferred into eppendorfs and pelleted at 13000 x g in a microfuge for 5 minutes at 4 °C and pellets were discarded and the supernatant collected. 30 µl Laemlli lysis buffer (appendix 2) was added to each western blotting lysate. Samples were stored at -20 °C. The wells of the gel were each loaded with 2-3 µl standard loading buffer and then 15 µl supernatant was loaded on a Bis-Tris NuPAGE pre cast polyacrylamide gel (Invitrogen) or a self cast gel (appendix 2). 5µl of a pre stained standard ladder was also loaded (Invitrogen). The experimental protocol is summarised in figure 2.1.
Figure 2.1: Flow diagram of experimental protocol for KK1 experiments

2.3.7 Primary human granulosa cells: Patients

Granulosa cells were obtained, with informed, written consent, at the time of egg collection for IVF at Hammersmith Hospital from 31 women described further in Chapter 4, section 4.3.1: 12 with normal ovaries (control), 8 with ovulatory PCOS (ov PCO) and 11 with anovulatory PCOS (anov PCO). These granulosa cells are isolated from the fluid that is collected from eggs in the ovary for IVF and are usually thrown away. Described in more detail in section 4.3 of Chapter 4. In this Chapter, two control patients were used to continue the work done in KK1 cells on androgen pre-incubation and insulin addition.
2.3.8 Human granulosa lutein cell collection, preparation and culture

All procedures were carried out under sterile conditions in a fume hood using aseptic tissue culture techniques. To enable removal of serum and reduce follicular fluid volume, samples were centrifuged at 200g for 5 minutes using a Centra CL3 (Thermo IEC) centrifuge. After discarding the clear follicular fluid supernatant, the pellets were then transferred into one 50mL centrifuge tube. If the volume of flush was too large, the pellets were centrifuged again and the process above repeated. The supernatant was then discarded and the cell solution volume was made up to 8mL with PBS.

In order to separate off any remaining red blood cells (RBCs) the follicular fluid mixture was gently layered over a percoll gradient. An autoclaved glass pipette was filled with cells and pipetted into 15mL centrifuge tubes containing 8mL pre-prepared shaken Percoll solution (8.9mL of Percoll [GE Healthcare], 1mL 10xM199, 100mL NaHCO₃ 7.5%, 10mL M199 [all from Invitrogen]). The 45% Percoll solution was filtered before use and shaken to produce bubbles, which provided a gentle surface for the cells to land on. The tubes were then centrifuged at 400g for 20-30min to isolate the granulosa-lutein cells; red blood cells separated off and sunk to the bottom of the tube. If the cell mixture was seen to be particularly bloody a greater number of test tubes of percoll gradients were used.

Granulosa lutein cells were then aspirated from the Percoll/PBS interface with an autoclaved glass pipette and washed twice, using serum supplemented
M199. Cell number and viability were determined using a Trypan Blue exclusion test (Sigma). Cell separation protocol summarised in figure 4.1.

For the glucose, lactate and progesterone assays, 20 x 10⁴ cells were plated into 96 well plates, and supplemented with 200µl serum supplemented medium 199 with or without DHT 10nM. Each experimental condition was repeated in triplicate. Cells were placed centrally and surrounding wells filled with sterile PBS to avoid evaporation. After 24h incubation at 37°C in 5% CO₂, serum containing medium was removed and the cells washed with PBS. They were then incubated for a further 24h with 200µL serum free Medium 199 with or without 100ng/ml of Insulin (Sigma) and DHT 10nM.

For the protein expression results, 50 x 10⁴ cells were added to 24 well plates and each well was supplemented with 1mL serum supplemented media +/- DHT 10nM. After 24h incubation at 37°C in 5% CO₂, the medium was removed and cells washed with PBS. They were then incubated for a further 24h with 1mL serum free Medium 199 +/-10nM DHT. The cells were then incubated with or without 100ng/ml of Insulin (Sigma) for 15, 30 or 60 minutes. Cells were then harvested in RIPA buffer, with aprotinin (MP Biochemicals, Cambridge, UK), protease inhibitors and sodium orthovanadate (Sigma). The samples were then centrifuged at 12000xg for 5 minutes at 4°C and supernatant collected. Laemmli sample suffer was added, and 20 µl loaded on a Bis-Tris NuPAGE pre cast polyacrylamide gel (Invitrogen). 5µl of a pre stained standard ladder was also loaded (Invitrogen) (see figure 4.2).
2.3.9 Western Blotting

Gels were run using a wet-blotting system, X cell Surelock Mini-Cell system. The gels were then transferred using a semi-dry transfer system. Polyvinylidene fluoride (PVDF) membrane (Thermo Scientific), was cut to the size of the gel and soaked in ethanol or methanol for membrane activation. Alternatively nitrocellulose membrane (Whatman) was used, which did not require prior activation. The transfer sandwich was then assembled in the following way: transfer sponge, followed by two sheets of Whatman chromatography paper, the PVDF membrane, the gel, a further two sheets of Whatman paper and finally another transfer sponge. The whole sandwich of layers was soaked in a dish filled with transfer buffer (appendix 2) and a roller was rolled over the sandwich to remove trapped air. The apparatus was closed into the transfer machine and proteins were then transferred for 3.5h at 200mA.

2.3.10 Immunoblot analysis

The membrane was first stained with ponceau to check protein transfer, and equal loading. The membrane was blocked with 5% skimmed milk and 1% 100mM sodium orthovanadate in 10% diluted 10x TBST buffer solution (Tris Buffered Saline with Tween) (blocking solution) for 15min at room temperature. Primary antibodies (appendix 1) were diluted in blocking solution and incubated with membranes overnight at 4°C. Blots were washed and incubated with the appropriate secondary antibody (appendix 1), for 1 hour at room temperature.
Signal was developed using ECL western blotting detection kit, and blots were then exposed to Kodak blue sensitive film. For the detection of total Akt and MAPK protein, membranes were stripped using stripping buffer (Thermo scientific) before re-probing. The following polyclonal antibodies were all obtained from New England biolabs (UK) LTD, Akt Ab, phospho-Akt (Ser473) Ab, phospho-Fox01 (Thr24)/Fox03a (Thr32), p42/44 phospho MAPK, p42/44 MAPK (appendix 1).

No quantitative analysis was performed as it was difficult on some occasions to scan, view and quantify the total protein blots with this method.

2.3.11 Measurement of lactate production and glucose uptake

Individual 3-µl aliquots of culture medium from the experimental and control incubation drops were collected using a 10µl pipette and diluted 1:200 for glucose analysis an 1:400 for lactate analysis, with a mixture of 5µM lactate and 2µM pyruvate solution. All samples were stored at -70°C until analysis with the COBAS bioanalyser.

Glucose and lactate assay

The depletion of glucose and the production of lactate by the granulosa cells were measured by analyzing the difference between substrate concentrations in the control and treatment wells as described previously (194). The assays were based on the conversion of substrate to product with the reduction of NAD⁺ (lactate assay) or NADP⁺ (glucose assay) (195). NADH and NADPH are highly fluorescent; hence the amount of fluorescence emitted is proportional to the amount of substrate in the sample. The fluorescence of the reactions was quantified using a COBAS Bio centrifugal autoanalyser (Roche Products,
Welwyn Garden City, UK) fitted with a fluorescence immunoassay (FIA) option. Reactions were carried out at 25°C. Light of a particular excitation wavelength (340nm) passed through the reaction mix in multiple cuvettes resulting in emission of secondary light. This is detected by a photomultiplier and signals were measured by photometer, and compared to a standard curve produced from 3 glucose or lactate standards (appendix 4). All chemicals were obtained from Roche (Welwyn Garden City, UK) unless otherwise stated. Cobas bioanalyser settings are set out in appendix 4. These programmes are based on those described by Stappenbeck et al. (196) and Harrison et al. (194, 197).

**Glucose assay**

Diluted media (1:200, 70µl) was automatically sampled and added to 200µl buffer containing 24ml Tris Buffer (0.1mM working concentration), 4.8ml MgCl2 Buffer (0.1mM), 500µl NATP (NADP 1.8mM and ATP 4.0mM). Hexokinase/glucose-6-phosphate dehydrogenase (40mM) diluted 1:80 with distilled water was added before fluorometric measurement.

**Hexokinase:** Glucose  + ATP ⇒ Glucose 6- phosphate + ADP

**G6PDH:** Glucose- 6 phosphate + NADP⁺ ⇒ 6-phosphogluconate + NADPH + H⁺

**Lactate assay**

Diluted medium (1:400, 40µl) was automatically sampled and added to 200µl buffer containing 0.54M Glycine (Sigma), 0.2M hydrazine sulphate (Sigma), 2.7nM EDTA (BDH). 0.54M sodium hydroxide (NaOH) and 0.6mM nicotinamide adenine dinucleotide (NAD). 20µl of lactate dehydrogenase (LDH) diluted 1:10 distilled water was added before fluorometric
measurement. The increase in fluorescence due to the formation of NADH is proportional to the amount of lactate consumed.

\[
\text{LDH: } \text{Lactate} + \text{NAD}^+ \rightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+
\]

**2.3.12 Immunocytochemistry (ICC)**

KK1 cells were plated onto a 24 well plate with a coverslip at the base of each well, at a density of \(1 \times 10^5\) cells per well. The cells were fixed with 4\% paraformaldehyde (Sigma) for 20 minutes at room temperature and then washed off 4 times with PBS. The cell membranes were then permeabilised with 0.1\% Triton. This was also washed 4 times with PBS. Each sample was blocked in the appropriate blocking serum for 20 minutes (20\% goat serum was made up using 1ml of inactivated goat serum, 0.2g bovine serum albumin (BSA) and 4ml PBS). The primary antibody was diluted in 20\% goat serum and samples were incubated overnight at 4\°C (appendix 1). The two primary antibodies used were, rabbit anti-GLUT4 antibody (Santa Cruz, California, USA) and rabbit anti-androgen receptor antibody (Fitzgerald: 20-RAR012, Acton, MA, USA). Some slides were incubated with rabbit immunoglobulin (IgG) (100\µg/ml) in PBS. One well was incubated in blocking solution alone with no primary antibody, thus acting as a negative control.

Each well was then rinsed three times with PBS before addition of biotinylated goat anti-rabbit IgG secondary antibody 1:200, for 30 min, then washed three times in PBS followed by application of peroxidase-conjugated avidin-biotin complex (Vector) for 30 minutes.

The well was then washed three times, followed by application of diaminobenzidine (DAB) (Zymed) for 10 minutes, after which the reaction was
terminated with distilled water. Haematoxylin was added for 30 seconds and then thoroughly washed off with water. The coverslips were then removed from the 24 well trays and mounted onto glass slides using Faramount (Dako, Glostrup, Denmark).

2.3.13 Transfection of KK1 cells with HA-GLUT4-GFP

2.3.13.1 Plasmid construction

HA-GLUT4-GFP was made from HA-tagged (Haemagglutinin tagged) human GLUT4 gene, pHA-GLUT4 vector (198) with enhanced GFP at the GLUT4 terminus in the GFP expression vector pQBI25 (Quatum Biotechnologies, Montreal, Canada) in two steps. The HindIII and KpnI restriction sites were initially inserted into the pQBI25 plasmid upstream of the GFP gene. The HA-GLUT4 sequence without the STOP codon was then inserted in between these restriction sites to yield pHA-GLUT4-GFP (199).

The KK1 cells were then transfected with an HA-GLUT4-GFP plasmid in order to assess sub-cellular trafficking (199) (kindly donated by J Tavare). The dual tagging of GFP and HA was created to aid differentiation of cell membrane and cytoplasm clearly in fat cells, but we have only used the GFP visualisation in the KK1 cells.

2.3.13.2 Elution of plasmid and transformation of DH5α cells with plasmid DNA

The HA-GLUT4-GFP plasmid was posted to our department on a piece of whatmann paper. This paper was soaked in distilled water overnight. To precipitate the DNA, 2µl of glycogen was then added together with 30µl 3M sodium acetate and 3x volume of 20°C 100% ethanol. This mixture was then
kept on ice for 1 hour followed by centrifuge for 30 minutes at 4\(^{\circ}\)C. 1mL 80% ethanol was then added. The supernatant was removed and the remaining pellet was left to air dry for 3 minutes. 20\(\mu\)l distilled water was then added, and left for 15 minutes on ice. After a quick spin to collect all the drops together, DNA was measured using a nanodrop machine.

Competent DH5 \(\alpha\) cells (Invitrogen) were transformed with 1-10ng of plasmid DNA. DNA was added to 100\(\mu\)l of cells and the tubes were placed on ice for 45 minutes before being heat shocked for 90 seconds at 37\(^{\circ}\)C. Cells were transferred to tubes containing 1 ml of 2 x TY and shaken for 30 minutes at 37\(^{\circ}\)C. Following this 100 \(\mu\)l of each culture was plated onto TY agar plates containing 100\(\mu\)g/ml ampicillin and incubated at 37\(^{\circ}\)C overnight in an inverted position.

The following day many colonies were seen on the agar plate. A tip was touched to an isolated large colony taking care not to touch the satellite colonies, and placed in a breathable test tube containing 2mls of TY and ampicillin at a concentration of 1:1000 (2\(\mu\)l). This test tube was then placed on a shaker at 250rpm at 37\(^{\circ}\)C overnight. The samples were then decanted into an eppendorf and spun for 30 seconds. The supernatant was removed and the QIAprep spin miniprep kit and protocol used on the pellet to amplify and purify up the plasmid DNA (Qiagen, West Sussex, UK).

### 2.3.13.3 Transient transfection

In preparation for these studies the KK1 cells were initially transfected with GFP alone, using a CMV promoter. During the optimisation process a GFP plasmid with H1 promoter was used, but the cells did not transfect well with
this. Different transfection methods were also tried such as calcium chloride and HBS, but the lipofectamine method worked best.

The cells were grown to 80% confluence and the growth media was changed to DMEM/F12 with glutamine 1%, and 10% FBS, but no antibiotic. Cells were transfected with lipofectamine lipid based transfection reagent as per manufacturers protocol (Invitrogen). Briefly, for transfection of plasmid DNA into a 6 well plate, 10 µl of lipofectamine was mixed with 240ul Opti-Mem I reduced serum media (Invitrogen) and in a separate tube, 240 µl of Opti-Mem I was mixed with 4 µg of plasmid. Each tube was mixed gently alone, and incubated for 5 minutes at room temperature. The two mixtures were then combined, mixed gently and incubated at room temperature for 20 minutes. The mixture was added to cells drop wise and incubated for 24-48 hours.

2.3.14 Live imaging using confocal microscopy

For live confocal microscopy, KK1 cells were plated at a density of 5 x 10^5 viable cells in 2ml of serum-supplemented DMEM-F12 (without phenol red or antibiotic) and incubated at 37°C in 5% CO₂. The KK1 cells were grown in a 35mm glass bottomed culture dish (Mat Tek Corporation, MA, USA), which combined the standard size 35mm disposable plastic petri dish with optical quality glass, providing high quality, high resolution microscopic images. The cells were grown to 80% confluence and were transiently transfected 24-48 hours in advance of the live confocal imaging (section 2.3.13.3). Prior to imaging, the KK1 cells were washed thoroughly with PBS and the medium was switched to serum free DMEM-F12 (without phenol red) for 24 hours. The cells were imaged without insulin for 10 minutes and then imaged for 10
minutes after insulin 100ng/ml addition with images taken at 10-second intervals. Fluorescence was observed using an SP5 confocal microscope. To excite GFP, the 488nm line of the argon laser was used at 10% laser energy. Each experimental condition was repeated in triplicate and the best examples are shown in figures 2.7 and 2.8.

2.3.15 Statistical analysis

Statistical analyses were performed using InStat 3.0a for Macintosh Graph Pad, San Diego, CA. Graphs were produced using GraphPad Prism Version 5.0. The experiments were repeated 2-3 times to overcome the fact that cell culture experiments can produce variable results on different occasions. However, the statistics were calculated using one experiment in which the cells were plated in triplicate. Non-parametric analysis was performed throughout as we were unable to calculate the normal distribution using three values.
Chapter 2

2.4 Results of KK1 cells

2.4.1 Effect of insulin on KK1 cells

2.4.1.1 Glucose uptake and lactate production

The uptake of glucose and the production of lactate was measured at 0, 15, 30, 60 minutes and 4, 24 and 48 hours after addition of 100ng/ml insulin. Dose response experiments were performed with 10ng/ml, 100ng/ml, and 1000ng/ml insulin, and glucose uptake and lactate production increased as the doses increased (data not shown). This particular insulin dose (100ng/ml) was chosen as although slightly supra-physiological it produced clear results. No significant changes were seen in glucose or lactate concentrations until after 48h in culture (Kruskal-Wallis non parametric anova, figure 2.2).

The effects of insulin were compared to those of incubation with medium alone. Control KK1 cells that had not been exposed to insulin, showed no change in glucose or lactate concentration at 48h. However a significant difference in glucose uptake was seen after 48h of insulin exposure compared to 48h of no insulin (p=<0.05, Mann Whitney). A significant difference in both glucose uptake and lactate production was also seen at 48h of insulin 100ng/ml incubation when compared to time zero (p=<0.05, Kruskal-Wallis, figure 2.3).

The experiment was repeated three times, however the statistics were calculated using one experiment in which the cells were plated in triplicate. Non parametric analysis was performed throughout it was not possible to calculate normal distribution using three values.
Figure 2.2: (a) Glucose uptake into KK1 cells after addition of insulin 100ng/ml. (b) Lactate production from KK1 cells after addition of insulin 100ng/ml. There were no significant changes in glucose or lactate levels until 48 hours. P values calculated using Kruskal-Wallis, non-parametric ANOVA. Results are of one representative experiment, cells plated in triplicate.
Figure 2.3: (a) Shows glucose depletion from the media, a significant difference was seen in glucose uptake after 48h of insulin exposure compared to 48h of no insulin (p<=0.05, Mann Whitney). (b) Lactate production after the addition of 100ng/ml insulin (grey bars) compared to KK1 cells with no insulin addition (control/green). A significant difference in lactate production was seen at 48h of insulin 100ng/ml incubation when compared to time zero (p<=0.05, Kruskal Wallis). No significant differences were seen when comparing 48h of insulin exposure vs 48hrs of no insulin exposure. Results are of one representative experiment, cells plated in triplicate.
Addition of a PI3K inhibitor LY294002 (LY) to the KK1 cells inhibited glucose uptake and lactate production. Using Kruskal-Wallis (non parametric one way anova) a significant reduction in lactate production was seen at both 24h and 48h of insulin exposure when compared to 24h (p=0.01) and 48h (p=<0.001) respectively of exposure to insulin after LY294002 25 μM pre-treatment (figure 2.4). No significant differences were seen in glucose uptake.

The experiment was repeated twice, however the statistics were calculated using one experiment in which the cells were plated in triplicate. Non parametric analysis was performed throughout as we were unable to calculate the normal distribution using three values.
Figure 2.4: Effect of PI3K inhibitor LY 294002: (a) Although glucose uptake was reduced after 48h of insulin 100ng/ml addition in the presence of 1h pre-incubation with PI3K inhibitor LY294002 25µM; no significant difference was seen (Kruskal-Wallis) (b) A significant reduction in lactate production was seen after 24h and 48h respectively of exposure to insulin after LY 294002 25 µM pre-treatment (Kruskal-Wallis). Results are of one representative experiment, cells plated in triplicate.
A second PI3K inhibitor wortmannin was used. One hour of either Wortmannin 1\(\mu\)mol \((p<0.001,\) Kruskal-Wallis\) or LY294002 25\(\mu\)M \((p<0.001,\) Kruskal-Wallis\) pre-incubation resulted in a significant reduction in lactate production in response to insulin 100ng/ml, although LY294002 produced a significantly greater inhibition than wortmannin \((p<0.001,\) Kruskal-Wallis\) (figure 2.5). The experiment was repeated twice, however the statistics were calculated using one experiment in which the cells were plated in triplicate. Non parametric analysis was performed throughout as we were unable to calculate the normal distribution using three values.
Figure 2.5: (a) Glucose uptake (b) lactate production in KK1 cells in response to two PI3K inhibitors LY and Wortmannin. Lactate production is significantly reduced in the presence of both wortmannin 1µmol (p=0.001) and LY 25 µM (p=0.001) after 48h of insulin 100ng/ml addition, compared to insulin treatment alone for 48h. The presence of 1h pre-incubation with PI3K inhibitor LY294002 25µM produced a significantly greater inhibition of lactate production from the medium than wortmannin 1µmol (p=0.001). Calculated using Kruskal Wallis. Results are of one representative experiment, cells plated in triplicate.
2.4.1.2 GLUT4 expression

GLUT4 expression was then explored in the KK1 cells. GLUT4 was shown to increase expression in response to insulin 100ng/ml (figure 2.6 b,c) but not forskolin 10µmol (figure 2.6 d).

The KK1 cells were then transfected with an HA-GLUT4-GFP plasmid in order to assess sub-cellular trafficking (199) (kindly donated by J Tavare). The dual tagging of GFP and HA was created to aid differentiation of cell membrane and cytoplasm clearly in fat cells, but we only used GFP visualisation in the KK1 cells.

Initially the cells were exposed to insulin and then mounted on slides and fluorescence imaging was used to analyse GLUT4 distribution prior to insulin addition. However it was not easy to monitor GLUT4 using this method (data not shown). Therefore live confocal imaging was used to visualise the GLUT4 response to insulin 100ng/ml as shown in figures 2.7 and 2.8. GLUT4 was seen to move toward the cell membrane after 5 minutes of insulin addition.
Figure 2.6: Immunocytochemistry: KK1 cells, GLUT4 (a) GLUT4 immuno-staining (primary antibody dilution 1:200) (b, c) GLUT4 immuno-staining (1:400 dilution) addition of insulin 100ng/ml for 1h showed GLUT4 up-regulation to the membrane (d) GLUT4 immuno-staining (1:400 dilution) addition of forskolin 10µmol for 1hr showed some background GLUT4 expression which was comparable to the control (a).
Figure 2.7: Live confocal imaging: HA-GLUT4-GFP transfected cells (a) GFP-GLUT4 transfected KK1 cell (b) GFP-GLUT4 transfected KK1 cell 5 minutes after insulin 100ng/ml addition, showed movement of GLUT4 towards the surface of the cell membrane.

Figure 2.8: Live confocal imaging: (a) GFP-GLUT4 transfected KK1 cell, 2 minutes after insulin 100ng/ml addition, note green tubular structure extending from cell, highlighted with an arrow (b) GFP-GLUT4 transfected KK1 cell, 5 minutes after insulin 100ng/ml addition, showed movement of GLUT4 expression and further green tubules, highlighted with an arrow.
2.4.1.3  Akt phosphorylation

The KK1 cells showed phosphorylation of Akt within 5 minutes of insulin 100ng/ml exposure. The expression of phosphorylated Akt increased with time and peaks at 60 minutes. Total Akt levels were used as a loading control and were unchanged throughout (figure 2.9). The blots of KK1 cell experiments were all imaged using ECL and exposed using Kodak blue sensitive film. No quantitative analysis was performed, as it was difficult on some occasions to scan, view and quantify the total protein blots with this method.

Insulin 100ng/ml

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Phosphorylated Akt

Akt

Figure 2.9:  Immunoblot; Time course of insulin 100ng/ml action on Akt phosphorylation. Western blot showing phosphorylated Akt, which was activated by 5 minutes and maximally stimulated at 1 hour. The membrane was stripped and re-probed for Akt as a loading control.
As the concentration of insulin changed from 10-1000ng/ml the amount of Akt phosphorylation also increased (figure 2.10). Some constitutive activation of Akt phosphorylation was seen at time zero.

Pre-incubation of PI3K inhibitor LY294002 (25µM) for 1 hour prior to insulin 100ng/ml addition, inhibited Akt phosphorylation (figure 2.11).

**Figure 2.10:** Immunoblot; Dose effect of insulin action on Akt phosphorylation. Phosphorylated Akt expression was shown to increase stepwise as insulin concentrations rise from 10-1000ng/ml.

**Figure 2.11:** Immunoblot; Effect of inhibition of PI3K on expression of phosphorylated Akt in KK1 cells. Akt phosphorylation was reduced with 1h LY294002 25µM pre-treatment.
2.4.1.4 MAPK phosphorylation

ERK also shows a time dependent rise in phosphorylation in response to insulin, but does not increase greatly with higher insulin concentrations (figure 2.12).

**Figure 2.12:** Immunoblot; Effect of insulin on MAPK. p42-44 ERK (MAPK) was phosphorylated in response to insulin (10-100ng/ml) and peaked at 15 minutes. There was no rise in expression with higher insulin concentrations.
2.4.2 Effect of androgen in KK1 cells

2.4.2.1 Immuno-cytochemistry

Expression of androgen receptors was confirmed in KK1 cells using immunocytochemistry (figure 2.13).

Figure 2.13: Immunocytochemistry: Expression of androgen receptor in KK1 cells: KK1 cells passage 10. Immuno-stained for IgG (control) and androgen receptor (primary antibody 1:50 dilution)
2.4.2.2 Glucose and Lactate

KK1 cells were then exposed to Dihydrotestosterone (DHT) 10nM alone. DHT 10nM alone produced a significant rise in lactate production \( (p<0.0019, \text{Kruskal-Wallis}) \) and significant glucose uptake at 48h \( (p<0.01, \text{Kruskal-Wallis} [\text{KW}]) \) compared to no treatment at 48h. Although this response was slightly attenuated by the anti-androgen Flutamide 1µmol, this result was not significant (figure 2.14).

**Figure 2.14**: Effect of DHT 10nM alone on glucose metabolism in KK1 cells: DHT 10nM alone produced an increase in lactate production \( (p<0.0019 \text{ KW}) \) and glucose depletion \( (p<0.01 \text{ KW}) \) from the media compared to no substrate addition at 48h. This response was partially attenuated by the presence of Flutamide 1µmol. Results are of one representative experiment, cells plated in triplicate.
Pre-incubation of KK1 cells with DHT 10nM followed by insulin 100ng/ml exposure, lead to a significantly higher depletion of glucose from media ($p<0.001$, Kruskal-Wallis), and a significantly higher lactate production at 48h ($p<0.01$, Kruskal-Wallis) than those cells not pre-incubated in androgen (figure 2.15).

**Figure 2.15:** Glucose uptake from the media and lactate production in KK1 cells exposed to both insulin and androgen. Pre-incubation of KK1 cells with DHT 10nM followed by insulin 100ng/ml exposure, lead to a significantly higher depletion of glucose from media ($p<0.001$), and a significantly higher lactate production at 48h ($p<0.01$) than those cells not pre-incubated in androgen. Kruskal-Wallis, one way anova was used for statistics. Results are of one representative experiment, cells plated in triplicate.
2.4.2.3 PI3K and MAPK Signalling

KK1 cells were then exposed to Dihydrotestosterone (DHT) 10nM alone and response of Akt phosphorylation and ERK phosphorylation monitored over time. KK1 cells were cultured in the absence of treatment and in the presence of DHT (10nM). Samples were collected at 5, 10, 15, 30 and 60 minutes and the phosphorylation of Akt and ERK was investigated (figure 2.16).

DHT 10nM

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Figure 2.16: Immunoblot, action of DHT 10nM alone in KK1 cells. KK1 cells, in the presence of DHT 10nM alone, phosphorylated Akt and ERK within 5 minutes, with a maximal stimulation at 15 minutes.
Insulin induced Akt phosphorylation was enhanced by 48h DHT 10nM pre-incubation (figure 2.17). This was also shown to be the case with longer periods of androgen exposure; 106h (figure 2.18) and 14 days (figure 2.17).

**Figure 2.17:** Immunoblot; effects of varying time lengths of androgen pre-exposure. Shows insulin induced Akt and ERK phosphorylation was enhanced in response to both short term (48hrs) and long term (14 days) androgen pre-incubation.

**Figure 2.18:** Pre-incubation with androgen for 106h, followed by insulin addition

a. Western blot showing the response of phosphorylated Akt and ERK to insulin (10-1000ng/ml) alone. The blot below shows total Akt and ERK used as a loading control.

b. Increased expression of phosphorylated Akt and ERK after 106hrs of DHT 10nM pre-incubation, followed by addition of insulin (10-1000ng/ml). The blot below shows total Akt and ERK as a loading control.
Pre-incubation with increasing doses of DHT (1, 10, and 25ng/ml), did not clearly produce an increase in the amount of phosphorylated Akt (figure 2.19). ERK phosphorylation also did not appear to increase with DHT concentrations. DHT 50ng/ml pre-incubation caused KK1 cell death.

Figure 2.19: Immunoblot showing DHT dose response. As the concentration of DHT used to pre-incubate the cells increases from 1nM to 25nM, the amount of phosphorylated Akt also rises. This is most evident at 30 and 60 minutes. ERK phosphorylation does not appear to increase with DHT concentrations.
2.4.2.4 Effect of androgen on human primary granulosa cells

In order to establish whether the results seen in the KK1 cells were limited only to this cell line, the experiments were repeated using human granulosa-lutein (GL) cells from two control patients with no evidence of PCOS. The results of these experiments mirrored those of the KK1 cells. DHT exposure for 48h enhanced insulin induced glucose uptake from the media and lactate production (figure 2.20) in primary human granulosa cells from 2 control patients (i.e without polycystic ovary syndrome, undergoing IVF for tubal infertility or sperm motility problems).

**Figure 2.20:** Human granulosa lutein cells from two control patients: Patient 1 and 2, glucose uptake and lactate production. A greater depletion of glucose from the media and lactate production was seen in the granulosa cells that have been exposed to 10nM DHT for 48hours before the addition of insulin 100ng/ml in both patients. This change was seen maximally at 48hours of insulin incubation and reached significance in the glucose uptake of Patient 1 but not Patient 2.
DHT pre-incubation also enhanced insulin induced phosphorylation of Akt in primary human GL cells from control patient 1, this is not so easy to see in control patient 2 [figure 2.21 (a) and (b)].

**Figure 2.21 (a)** Human granulosa lutein cells from control patient, immunoblot: Patient 1, Effect of androgen on insulin action in control human granulosa cells. Western Blot showing increased phosphorylated Akt when cells from control women were pre-incubated for 48hours in DHT 10nM prior to the addition of insulin 100ng/ml.

**Figure 2.21 (b):** Human granulosa cells from control patient, immunoblot: Patient 2, Western Blot showing increased phosphorylated Akt when cells from patient 2 were pre-incubated for 48hrs in DHT 10nM prior to the addition of insulin.
2.5 Discussion

2.5.1 Effect of insulin in KK1 cells

As in other insulin sensitive tissues such as adipose (188) and skeletal muscles (200), Akt and ERK phosphorylation in KK1 granulosa cells responded to insulin in a dose and concentration dependent manner.

Using LY294002 and wortmannin, we have shown PI3K inhibition leads to impaired glucose uptake and metabolism, this was associated with impairment of insulin stimulated Akt phosphorylation, with no effect on insulin stimulated MAPK phosphorylation. This suggested that the PI3K pathway is important in glucose metabolism in KK1 cells. This is supported by studies in primary porcine granulosa cells that have also shown that wortmannin prevents insulin induced Akt phosphorylation and glucose uptake (201). This study used mRNA expression to show that the induction of insulin resistance using wortmannin, led to a reduction in GLUT4 mRNA and an increase in MAPK mRNA.

We have also shown that GLUT4 was up-regulated to the cell membrane in response to insulin using immunocytochemistry. Live confocal imaging has allowed visualisation in real time of how GLUT4 constantly cycles from an intracellular compartment up to the cell surface and back down again within a few minutes of insulin exposure.

2.5.2 Effect of insulin and androgen

The most significant finding in this study was that exposure to androgen in vitro both in the short [48-106h] and long term [14 days] enhanced rather than
inhibited insulin-stimulated phosphorylation of Akt as well as glucose uptake and metabolism. This was reproduced in primary human granulosa cells.

Interestingly, DHT alone also augmented Akt and ERK phosphorylation. Uptake of glucose from the media and production of lactate was highest in response to DHT (10nM) compared to both treatment with insulin 100ng/ml, and intrinsic glucose uptake and lactate production with no stimulation. Flutamide (1µmol), an androgen receptor antagonist, partially attenuated this response, thus supporting the effect of DHT alone. It also suggested that this response is stimulated via the AR, which we have demonstrated in KK1 cells using immunocytochemistry. This effect of insulin and androgen augmentation on glucose metabolism and Akt phosphorylation was also seen in two experiments done using primary human granulosa cells from 'control' women without polycystic ovary syndrome.

Rather than the expected inhibition of insulin-stimulated glucose uptake and lactate production, this augmentation by androgens is a surprising result. The results suggest that the insulin resistance observed in granulosa cells from women with PCOS (71), can not be explained by direct exposure to androgen. The actions of DHT are particularly interesting as although testosterone can be aromatised to oestradiol by the enzyme aromatase, DHT, which is an irreversible end product of 5 α-reductase action on testosterone, can not.

Our results correlate well with some recent work done in skeletal muscle. It has been shown that T and DHEA activate glucose metabolism-related signalling in skeletal muscle. The addition of T or DHEA to cultured primary rat skeletal muscle cells increased GLUT4 protein expression, phosphorylation of Akt and protein kinase C-ζ/λ, and also increased activity of
hexokinase and phosphofructokinase which are important glycolytic enzymes (202). It has also been shown in osteoblasts that testosterone accelerates Akt phosphorylation via the PI3K pathway, and that this effect is abolished using a PI3K inhibitor (203).

However our results contrast with work done in adipocytes. Corbould et al. investigated the action of androgen on pre-adipocytes from healthy women treated with testosterone or anti-androgens for 48h. Maximal insulin stimulated glucose uptake (insulin 10nM) was impaired in the cells treated with testosterone 10nmol/L and 100nmol/L. This defect was attenuated by co-incubation with anti-androgens. However testosterone treatment did not alter phosphorylation of insulin receptor substrate-1, Akt or MAPK. It did however impair atypical protein kinase C (PKC) isoform $\zeta$ phosphorylation (188). PKC is activated by PI3K and PDK, and has been shown to be important in glucose metabolism (204). These different results may be due to tissue specific differences in insulin action on glucose metabolism, this has previously been reported in PCOS (131).

Work done by Depi Bessi an MSc student in our laboratory using quantitative polymerase chain reaction, has shown that insulin up-regulates androgen receptor (AR) expression in passage number ten KK1 cells after 24h of treatment (unpublished). This result is consistent with findings in skeletal muscle cells which revealed that the insulin-like growth factor-I is able to induce AR gene expression (176). Preliminary data, using relative luciferase activity measurements in HeLa cells after co-incubation with androgen and insulin showed that insulin potentiates the effect of androgens by inducing AR gene expression (Patel et al., Endocrine Society annual conference, 2010,
abstract P1-350). Other evidence for cross talk between androgen signalling systems and growth factor/tyrosine kinase pathways comes from a study by Reinikainen et al. 1996, again in this experiment HeLa cells were co-transfected with androgen receptor expression vector, and exposure to EGF and IGF1 increased AR dependent transactivation by 1.5 to 2.5 fold (205).

Insulin like growth factor 1 (IGF-1) activates both AR serine phosphorylation and AR translocation into the nucleus of skeletal muscle cells, via PI3K and Akt phosphorylation. An increase in AR target gene expression (skeletal α-actin) was also seen, suggesting that rapid non-genomic signalling may lead to an increase in gene transcription and translocation (176). One other mechanism of interaction between androgen and insulin could be via Forkhead transcription factor (FOXO-1 or FKHR). Liganded AR recruits FOXO1 to the AR promoter area, where it interferes with AR-DNA interactions. It acts as a co-repressor of AR. Insulin and insulin like growth factor 1 (IGF1) abolish this occupancy, and relieve this inhibition (87). PI3K signalling which is activated by both insulin and IGF1 phosphorylates FOXO1. The phosphorylated FOXO1 is inactive and expelled from the nucleus (88).

In some western blots a constitutive activation of protein phosphorylation, especially of Akt, is seen in KK1 cells. One explanation could be that KK1 cells were created using Simian Virus 40 (SV40) T-antigen transgene and it has been reported that this T-antigen can bind to IRS1 and therefore activate Akt (206, 207).

One anomaly noted was that glucose uptake from the media and lactate production, were only detected at 24-48h of exposure to substrates such as insulin although phosphorylation of Akt and ERK and also movement of
GLUT4 were seen within minutes. It was postulated that the COBAS machine was insensitive to small amounts of glucose uptake, and if time had permitted it was thought that the use of a second method to measure glucose uptake such as measurement of the uptake of radiolabelled glucose from the media would have been a sensitive method of monitoring small amounts of glucose taken up rapidly by KK1 cells.
Chapter 3

Effects of FSH on glucose metabolic pathways in KK1 cells; interaction with androgen
3.1 Introduction

3.1.1 Two cell two gonadotrophin concept

In women, androgen is the main steroid made by thecal cells, and aromatase activity is highest in granulosa cells (39). It is generally assumed that interaction occurs between the two cell types in the follicle with LH stimulating androgen production from the thecal cells, which is then used by the granulosa cells to produce oestrogen, this is known as the two-cell two-gonadotrophin concept (figure 1.3).

3.1.2 Gonadotrophin signalling in the ovary

The response of granulosa cells to LH and FSH is mainly mediated by cyclic adenosine monophosphate/ protein kinase A (cAMP/PKA) signalling. LH binds the LH receptor and FSH binds the FSH receptor both of which are transmembrane receptors. On binding the gonadotrophins both receptors stimulate the Gs protein (144). GDP bound to the αGs subunit is exchanged for GTP and the αGs-GTP subunit breaks off and binds with adenylate cyclase. This generates cAMP, which then binds the regulatory units of PKA. The complex then dissociates into 2 catalytic subunits and a regulatory subunit. The catalytic subunit then phosphorylates cAMP response binding proteins (CREM and CREB), which bind upstream DNA regulatory elements called cAMP response elements. Genes such as those encoding the LH receptor and P450 aromatase are regulated by this method (figure 3.1).
Figure 3.1: PKA signalling. FSH binds to its receptor, causing $G_{so}$ subunit dissociation. Together with GTP, this complex directly activates adenylate cyclase (AC), leading to cAMP synthesis. PKA is activated by cAMP, which causes the dissociation of the catalytic subunit (C) from the regulatory subunit (R). The catalytic site can activate proteins by phosphorylation. cAMP production also leads to an intracellular rise of Ca^{2+}. In the nucleus the catalytic subunit of the PKA phosphorylates (P) and activates transcription factors such as CREB and CREM, which then bind to CREs (cAMP response elements) preceding certain genes. Finally mRNA synthesis of primary response genes of FSH begins. Adapted from Simoni et al. 1997 (208).

3.1.3 Gonadotrophin activation of other signalling pathways

LH and FSH are classically believed to act via the PKA pathway, but there is evidence that other pathways such as the PKB and MAPK pathway are also stimulated by gonadotrophins (209, 210), illustrated in figure 3.2. Cyclic AMP (3',5' cyclic adenosine monophosphate) is a second messenger and induces physiological responses such as growth and gene expression. These effects have been attributed to the binding of cAMP to cAMP-dependent protein kinase A (PKA). However cyclic AMP may act to control diverse cellular
signalling pathways. A family of cAMP-binding proteins that exhibit both cAMP-binding and guanine nucleotide exchange factor (GEF) domains has been seen. These cAMP-regulated GEFs (cAMP-GEFs) bind cAMP and selectively activate the Ras superfamily guanine nucleotide binding protein Rap1A in a cAMP-dependent but PKA-independent manner (211, 212). cAMP-GEFI has been shown to regulate the activity of Rap1, which can activate B-Raf kinase, leading to activation of the ERK pathway.

These cAMP-GEFs, by activation of Rap-1 and possibly other GTPases, are potential activators of PI3-K, a known target of Ras (213). Thus, cAMP may regulate specific members of the PKB and MAPK pathways by PKA-independent as well as PKA-dependent mechanisms (214).

FSH also activates serum and glucocorticoid inducible kinase (SgK), a member of the serine threonine protein kinase family. It is also a downstream target of PI3K in addition to PDK (215).
Figure 3.2: Follicle-stimulating hormone (FSH) and insulin/insulin-like growth factor-1 (IGF-1) signalling pathways operate in granulosa cells. FSH/LH and IGF-1 stimulate the proliferation and differentiation of granulosa cells. Common downstream targets of FSH and IGF-1 in granulosa cells include components of the PI3-kinase cascade that lead to the phosphorylation of protein kinase B (PKB) and serum and glucocorticoid-induced kinase (Sgk) as well as the transcription factor, Forkhead (FKHR), which is selectively expressed at high levels in granulosa cells of growing follicles. Adapted from Richards et al. 2002 (216).

Gonadotrophins are also believed to enhance the activity of the p38 MAPK (139), and ERK pathways (140), as well as the PI3K (141) pathway, both pathways being involved in glucose metabolism and uptake (70, 142). PKA selective inhibitors inhibit the activation of these signalling pathways and target genes, suggesting a PKA dependent mechanism of PI3K and ERK activation (140, 143). The FSH mediated increase in cAMP has also resulted in up-regulation of the PI3K pathway (141, 214). Other evidence that FSH activates the PI3K pathway includes the fact that a PI3-kinase inhibitor LY294002 significantly decreases FSH stimulated glucose uptake in mouse cumulus oocyte complexes (70).
3.1.4 **Role of gonadotrophins in energy metabolism**

Oocytes depend primarily on energy metabolites derived from granulosa cells to allow growth and protein synthesis (92, 217, 218). Most studies concur that mouse ovarian follicles produce large amounts of lactate during growth and development and prefer a predominantly glycolytic mode of energy production to sustain growth and steroidogenesis. The rate of glycolysis is controlled by gonadotrophins. The amount of lactate produced suggests that ovarian follicles behave like solid tumours, predominantly metabolising glucose via glycolysis (98). This is supported by the observation that when oxidative phosphorylation is prevented using a TCA cycle inhibitor, follicles are still able to undergo normal development and steroidogenesis (99).

The average glucose concentration of follicular fluid is 3.29mM, compared to a plasma glucose concentration of about 5mM (93). Human serum lactate concentrations range between 1.5-2mM (94), considerably lower than the average lactate level in follicular fluid of 6.12mM (93), which suggests lactate is produced locally in the follicle and secreted into follicular fluid. This is supported by the fact that gonadotrophins are known to stimulate glucose consumption and lactate production by cumulus cells (95). FSH also induces cumulus cell-enclosed mouse oocyte expression of the glycolytic enzyme hexokinase (96).

In human granulosa-lutein cells, LH has been shown to have a dose dependent effect on lactate production in both women with normal and polycystic ovaries as shown in Figure 1.12 (71).
Chapter 3

3.2 Aim

Studies from Chapter 2 have shown the effect of insulin and androgen on PI3K and MAPK pathway activation, as well as glucose metabolism in KK1 cells.

The effects of FSH on glucose metabolism and activation of the PI3K and MAPK pathways (and the interaction with androgen) was studied using KK1 cells transfected with the FSH receptor.

Specific aims were:

1. to explore the effect of FSH on glucose uptake and metabolism
2. to define the signalling pathways involved and to investigate the effect of androgen, on FSH mediated signalling
3.3 Materials and Methods

3.3.1 Transfection of KK1 cells with the FSH receptor

KK1 cells are generally poorly responsive to gonadotrophins. FSH receptor expression is typically maintained for up to 10 passages while LH receptor expression is typically maintained up to 20 passages (189). However, if the FSH receptor is transfected into KK1 cells, it can become a suitable granulosa cell line to demonstrate the effects of FSH and androgen on glucose metabolism of granulosa cells.

A vial of frozen KK1 cells, which had previously been stably transfected with a plasmid expressing human FSH receptor (FSH-R) using the lipofection method, was used for the FSH induction experiments. The construction of the wild type human FSH-R expression plasmids is described by Aittomaki et al. 1995, and Gromoli et al. 1992 (219, 220). FSH receptor plasmid transfection was achieved with a total of 6µg of expression plasmid with 0.6µg of neomycin resistance plasmid pPGKneobpA. After transfection, resistant colonies were picked, expanded and screened for FSH receptor mRNA (221). The lipofectamine transfection method is described in section 2.3.13.3.

3.3.2 Cell culture and splitting cells

Cells were revived from frozen cell stocks as described in section 2.3.2. All culture dishes (Nunc, VWR) and flasks (TPP, Helena Biosciences) were suitable for tissue culture and all serological plugged pipettes were purchased from Costar. All experiments were carried out in a Class II flow cabinet and cells were grown at 37°C in 5% CO₂. KK1 cells were grown in Dulbeco’s modified medium (DMEM-F12) supplemented with 10% heat inactivated foetal
calf serum (FCS), 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 μM glutamine (Invitrogen, Gibco). Cells were passaged thrice weekly by incubating with 5 ml of 0.25% trypsin/EDTA mix (T3924, Sigma) for 3 minutes. The cells were re-suspended in 5 ml of DMEM-F12 supplemented with 10% heat inactivated FCS, 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 μM glutamine (Invitrogen, Gibco) and plated to the required density. Cells were grown in T75 flasks until confluent and some were frozen down as described in section 2.3.4.

3.3.3 Cell counting

Cells were counted using disposable haemocytometers (Hycor Biomed) using a light microscope (Olympus CK2). The experiments were repeated 2-3 times to overcome the fact that cell culture experiments can produce variable results on different occasions. For each experiment the KK1 cells were plated in triplicate.

3.3.4 Cell culture, protein isolation and expression

For protein expression experiments, cells were cultured in 6 well plates at a density of 1.4 x 10^6 cells/well, in 3mls of serum-supplemented medium 199. For glucose uptake and metabolism, cells were plated in 96 well plates at a density of 2 x 10^4 viable cells/well in 200μl of serum-supplemented medium 199. For each experiment, the KK1 cells were plated in triplicate. To those KK1 cells being exposed to androgen, Dihydrotestosterone (DHT) was added at this stage. The DHT was dissolved in ethanol (appendix 3), and the ethanol was tested to ensure it had no effects at the concentrations used. After 24 hours incubation at 37°C in 5% CO₂, the medium was removed and cells
washed thoroughly with PBS. They were then incubated in serum free DMEM-F12 +/- 10nM DHT (appendix 3). FSH 20ng/ml (dissolved in PBS +0.1% bovine serum albumin) was added for various amounts of time (appendix 3). Cells were lysed in 100 µl ice cold RIPA buffer directly on the plate, with 10 µl protease inhibitors, 10 µl aprotinin (MP Biochemicals) and 10 µl sodium orthovanadate. Using a cell scraper, the lysates were transferred into eppendorfs and pelleted at 13 000 x g in a microfuge for 5 minutes at 4 °C and pellets were discarded and the supernatant collected. 30 µl Laemmli lysis buffer (see appendix 2) was added to each western blotting lysate. Samples were stored at -20 °C. The wells of the gel were each loaded with 2-3 µl standard loading buffer and then 15 µl supernatant was loaded on a Bis-Tris NuPAGE pre cast polyacrylamide gel (Invitrogen) or a self cast gel (appendix 2). 5µl of a pre stained standard ladder was also loaded (Invitrogen).

3.3.5 Western Blotting

Gels were run using a wet-blotting system, X cell Surelock Mini-Cell system. The gels were then transferred using a semi-dry transfer system. Polyvinylidene fluoride (PVDF) membrane (Thermo Scientific), was cut to the size of the gel and soaked in ethanol or methanol for membrane activation. Alternatively nitrocellulose membrane (Whatman) was used, which did not require prior activation. The transfer sandwich was then assembled in the following way: transfer sponge, followed by two sheets of whatman chromatography paper, the PVDF membrane, the gel, a further two sheets of whatman and finally another transfer sponge. The whole sandwich of layers was soaked in a dish filled with transfer buffer and a roller was rolled over the
sandwich to remove trapped air. The apparatus was closed into the transfer machine and proteins were then transferred for 3.5h at 200mA.

### 3.3.6 Immunoblot analysis

The membrane was first stained with ponceau to check protein transfer, and equal loading. This was then washed off with water. The membrane was blocked with 5% skimmed milk and 1% 100mM sodium orthovanadate in 10% diluted 10x TBST buffer solution (Tris Buffered Saline with Tween) (blocking solution) for 15min at room temperature. Primary antibodies (appendix 1) were diluted in blocking solution and incubated with membranes overnight at 4°C. Blots were washed and incubated with the appropriate secondary antibody (appendix 1), for 1 hour at room temperature. Signal was developed using ECL western blotting detection kit, and blots were then exposed to Kodak blue sensitive film. For the detection of total AKT and MAPK protein, membranes were stripped before re-probing. The following polyclonal antibodies were all obtained from New England biolabs (UK) LTD, Akt Ab, phospho-Akt (Ser473) Ab, phospho-Fox01 (Thr24)/Fox03a (Thr32), p42/44 phospho MAPK, p42/44 MAPK.

### 3.3.7 Measurement of lactate production and glucose uptake

Individual 3-µl aliquots of culture medium from the experimental and control incubation drops were collected using a 10µl pipette and diluted 1:200 for glucose analysis an 1:400 for lactate analysis, with a 5µM lactate and 2µM pyruvate solution. All samples were stored at -70°C until analysis with the COBAS bioanalyser as described in section 2.3.11.
3.3.8 Progesterone Measurements

Progesterone levels in the spent medium were determined using the Abbott ARCHITECT i2000w automated Analyzer, which uses a chemiluminescent immunoassay and measures progesterone in nanomolar concentrations. Individual 3-µl aliquots of culture medium from the experimental and control incubation drops were collected using a 10µl pipette and diluted 1:100 with serum free medium 199. Assays were performed in the Clinical Chemistry Laboratory, Hammersmith Hospital with the help of Dr Mandy Donaldson.

3.3.9 Statistical analysis

Statistical analyses were performed using InStat 3.0a for Macintosh Graph Pad, San Diego, CA. Graphs were produced using GraphPad Prism Version 5.0. The experiments were repeated 2-3 times to overcome the fact that cell culture experiments can produce variable results on different occasions. However the statistics were calculated using one experiment in which the cells were plated in triplicate. Non parametric analysis was performed throughout as we were unable to calculate the normal distribution using three values.
Chapter 3

3.4 Results

3.4.1 Effects of FSH and androgens on glucose metabolism

Addition of FSH 20ng/ml to KK1 cells transfected with the FSH receptor did not significantly increase either glucose uptake from the medium or lactate production. DHT 10nM alone as before did increase glucose metabolism significantly (<0.01, Kruskal-Wallis). But the addition of FSH did not augment the response to DHT (figure 3.3).

**Figure 3.3: Effect of FSH and androgen on glucose uptake and metabolism.** Addition of FSH 20ng/ml to KK1 cells transfected with the FSH receptor did not significantly increase glucose uptake from the medium or lactate production. DHT 10nM alone produced a significantly higher glucose uptake from the medium (p<0.01, KW) and lactate production (p<0.01, KW). FSH 20ng/ml did not augment the effect of DHT.
3.4.2 Effects of FSH and androgen on glucose metabolic pathways

Akt was phosphorylated in response to FSH 20ng/ml (dose chosen after dose response experiments shown in figure 3.7 and 3.8). It was activated within 15 minutes. Pre-incubation for 1 hour with the PI3K inhibitor, LY294002, appropriately inhibited this response (figure 3.4). Of interest is that phosphorylated Akt at time zero was also abolished by LY294002 which suggests that there is some constitutive Akt phosphorylation in the absence of FSH.

![Phosphorylated-Akt](Figure 3.4)

**Figure 3.4**: Immunoblot: FSH 20ng/ml effect on PI3K. KK1 cells transfected with the FSH receptor. FSH produced a time dependent increase in phosphorylated Akt which was appropriately inhibited by PI3K inhibitor LY294002.
FSH 20ng/ml also activated the MAPK pathway in a time dependent manner (figure 3.5), as expected this response was not affected by PI3K inhibitor LY294002 (figure 3.6).

![Figure 3.5: Immunoblot, FSH 20ng/ml effect on MAPK. KK1 cells transfected with the FSH receptor demonstrated a time dependent increase in ERK phosphorylation in response to FSH 20ng/ml.](image1)

![Figure 3.6: Immunoblot, FSH effect on MAPK unaffected by LY294002. KK1 cells transfected with the FSH receptor demonstrated a time dependent activation of phosphorylated ERK in response to FSH 20ng/ml, which as expected was unaffected by the presence of an inhibitor of the PI3K pathway LY294002](image2)
Two doses of FSH were compared, although there may be a slightly greater amount of Akt phosphorylation (figure 3.7) and a greater amount of ERK phosphorylation (figure 3.8) at FSH 20ng/ml compared to FSH 2ng/ml. This is not very clear from our immunoblots.

**Figure 3.7:** Immunoblot; Effect of different FSH concentrations on PI3K. In KK1 cells transfected with the FSH receptor, two FSH concentrations were compared; 2ng/ml and 20ng/ml. It is not clear if FSH 20ng/ml produced a greater amount of Akt phosphorylation compared to FSH 2ng/ml.

**Figure 3.8:** Immunoblot; Effect of different FSH concentrations on MAPK. In KK1 cells transfected with the FSH receptor, FSH 2ng/ml produced no detectable effect. FSH 20ng/ml stimulated phosphorylation of ERK.
Unlike the results from Chapter 2, which showed insulin and androgen together had a synergistic effect on PI3K activation and Akt phosphorylation, the action of FSH 20ng/ml on both Akt and ERK phosphorylation was unaffected by DHT (10nM) pre-incubation for 48 hours (figure 3.9, experiments performed by Pavidra Sivanandarajah, BSc student, under supervision).

**Figure 3.9:** Immunoblot; FSH induced signalling in response to androgen pre-incubation. (a) Akt was phosphorylated in response to FSH 20ng/ml in a time dependent manner. It was up-regulated by 5 minutes and continues to be stimulated at 60 minutes. This is not affected by DHT (10nM) pre-incubation for 48h. (b) ERK was maximally stimulated by 15 minutes.

**Progesterone** concentration in the medium measured in nmol/L was undetectable in the presence or absence of FSH 20ng/ml +/- DHT 10nM pre-incubation (results not shown).
3.5 Discussion

FSH did not increase glucose metabolism significantly in these experiments, but nevertheless did increase phosphorylated Akt in a time and dose dependent manner. This may be due to a failure of KK1 cell transfection however this seems unlikely given Akt was phosphorylated in response to FSH. It may be to do with FSH receptor transfection density, this was not visualised using immunocytochemistry. Or there may be a minimal effect on glucose metabolism that has not been picked up using the COBAS bioanalyser. Different doses of FSH were used in glucose metabolism but no significant differences were seen (data not shown). Interestingly, and in contrast to the effect of insulin action, pre-incubation with DHT had no significant effect on FSH stimulated PI3K or MAPK phosphorylation or glucose metabolism.

Previous studies have investigated lactate accumulation but not glucose uptake of granulosa cells in the rat (95) and in humans (94). Unlike the results of our study, androgen was shown to enhance FSH stimulated lactate production after 48h of incubation in rat granulosa cells (95). Androgen and FSH had a synergistic effect on lactate accumulation, but androgen had no effect alone. However, the doses used in our study were physiological, while the doses used in the rat study were 5 fold higher (100ng/ml) for FSH and 10 fold higher (100nM) for DHT.

Harlow et al. used primary human granulosa cells exposed to either FSH 30ng/ml alone or FSH 30ng/ml and testosterone 0.1µM, to assess lactate accumulation and therefore a measure of glycolysis. FSH significantly increased glucose metabolism of human granulosa cells prior to the LH surge.
The effects of androgen did not give clear results. Testosterone 0.1 µM was only shown to have a synergistic effect with FSH on glucose metabolism of granulosa cells from one out of three subjects prior to LH surge, but no response was seen in granulosa cells from post the LH surge (94).

In certain experiments a constitutive activation of Akt phosphorylation is present. This could be because KK1 cells were created using Simian Virus 40 (SV40) T-antigen transgene and it has been reported that this T-antigen can bind to IRS1 and thus activate Akt (206, 207).

We have demonstrated FSH 20ng/ml induces MAPK phosphorylation, as judged by an increase in phosphorylated ERK within 5 minutes, and peak at 15 minutes. This is supported by other data showing FSH stimulates phosphorylation of MAPK in rat granulosa cells, with peak activity between 10-20 minutes after FSH addition before decreasing (140). One of the limitations of our study includes the fact that we cannot correlate this MAPK phosphorylation with progesterone production.

Multiple attempts were made to measure progesterone production in KK1 cells. Previous reports of progesterone production results were obtained using cells of passage 8 which were unavailable to us (189). Our experiments using KK1 cells of passage 10 or higher showed undetectable or very low progesterone levels in response to FSH 20ng/ml, insulin 100ng/ml and DHT 10nM. This suggests a de-differentiation of KK1 cells with respect to steroidogenesis at this passage level. Although it may also be due to the fact that the Abbott analyser, which is used clinically in the biochemistry department of Hammersmith hospital, measures progesterone concentrations...
in the blood in nanomolar concentrations, which was not sensitive enough to detect the amounts produced by the KK1 cells.

Prior to receiving the FSH receptor transfected cells some initial experiments were done using forskolin (data not shown). Forskolin activates cAMP and therefore acts to mimic the action of LH and FSH on the PKA pathway. Phosphorylated Akt and ERK are activated by both insulin 100ng/ml and forskolin 10\(\mu\)M in a time dependent manner. It appears that the addition of insulin and forskolin together produces an augmented response in phosphorylated Akt expression. Phosphorylated FOXO 03/01, a downstream marker of PI3K pathway is activated in a similar pattern to AKT phosphorylation but after a short time delay. Forskolin did not however activate glucose uptake or metabolism.

The main disadvantage of working with the KK1 cell line was the likelihood of changing phenotype over time as the passage number grows. As always with cell line work it is important to appreciate that we are unsure how closely these KK1 cells represent primary mouse granulosa cells. But they provide a good model on which to experiment.

Overall transfected KK1 cells are not ideal for studying the effect of FSH on glucose metabolism, there is a poor response compared to primary cells, but there is an effect of FSH on signalling, albeit unaffected by androgen. These experiments could be repeated using primary human granulosa cells from women without PCOS (control patients), as in Chapter 2 to confirm whether or not an effect is present.
Chapter 4

Defining insulin signalling pathways in primary granulosa-lutein cells in women with polycystic ovary syndrome
4.1 Introduction

4.1.1 Insulin resistance, mechanisms and complications

Insulin resistance is an inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilisation as much as it does in the normal population. The main consequences are a reduced ability of insulin to suppress hepatic gluconeogenesis and stimulate peripheral glucose uptake. It would be expected that circulating glucose levels would rise in this state of insulin resistance, however provided β cell function of the pancreas is adequate, insulin secretion increases to overcome this insulin resistance and glucose levels are normalized. Compensatory high insulin levels are therefore the hallmark of insulin resistance. Insulin resistance plays a major role in the development of type 2 diabetes, but it is important to note that other factors such as defective insulin secretion play an equally important role.

In the UK 4.26% of the population have diagnosed diabetes (Quality and Outcomes Framework 2010) and an estimated further 3.1 % of men and 1.5% of women have undiagnosed diabetes (Health Survey for England 2003). It is estimated that by 2020 there will be 250 million people affected by type 2 diabetes mellitus worldwide (222).

Type 2 diabetes has many complications including cardiovascular disease such as stroke and myocardial infarction and also microvascular disease such as retinopathy, nephropathy and neuropathy. Women with PCOS have reduced insulin sensitivity (11) and a 3-4 times greater risk of developing type 2 Diabetes (119, 120). Insulin resistance and subsequent hyperinsulinaemia
are found in 50-70% of PCOS women (6). Interestingly insulin resistance is most prevalent in women with PCOS who have both androgen excess and anovulation. Whereas weight matched women with ovulatory cycles, who are equally hyperandrogenaemic have normal insulin sensitivity and serum insulin concentrations (figure 1.10).

Insulin resistance has been implicated in the mechanism of anovulation seen in PCOS patients. Therapies that lower insulin levels such as insulin sensitising drugs and weight loss significantly improve menstrual cyclicity, fertility, and hyperandrogenism, in both lean and obese patients, which suggests insulin resistance plays an important role in the aetiology of anovulation in PCOS (122) (123).

**Mechanism of insulin resistance in PCOS**

The activation of the PI3K signalling pathway by insulin stimulates the metabolic functions of insulin, such as glucose uptake and glycogen synthesis. However there is a divergence of the insulin signalling pathway following activation of the insulin receptor. The insulin receptor once activated can activate the MAPK signalling pathway, which stimulates certain mitogenic actions of insulin such as cell metabolism, growth, differentiation and steroidogenesis (figure 1.8). Insulin resistance in PCOS appears to be selective affecting metabolic but not mitogenic activity of insulin (71, 72). In insulin resistant states when pancreatic β cells are continuing to compensate, insulin levels rise overcoming insulin resistance and maintaining glucose levels. However the hyperinsulinaemia can lead to an overstimulation of mitogenic actions. This selective insulin resistance may explain how insulin acting through the insulin receptor can stimulate the overproduction of
androgen from thecal cells in women with PCOS, despite resistance to the
effect of insulin on glucose metabolism (15, 73). MAPK activity is not reduced
in type 2 diabetes, and it has been suggested that this may provide a
mechanism for some of the detrimental effects of chronic high insulin levels
on cell growth in vessels (104, 105).

The exact mechanism of reduced insulin sensitivity in PCOS is unknown as
explained in section 1.8.5 of Chapter 1. Potential areas of disturbance in the
insulin signalling pathway are highlighted in Table 1, adapted from Diamanti-
Kandarakis et al. (124).

Abnormalities of the insulin receptor itself are rare causes of PCOS and much
evidence points to a post-receptor defect in insulin signalling pathways (3, 7,
127). It is likely that the signalling defect in insulin resistance seen in PCOS is
located proximally in the pathway involving serine phosphorylation of the
insulin receptor (IR) and/or insulin receptor substrates 1 and 2 (IRS1/2) (3,
127).

Another feature of insulin target tissues in women with PCOS is that
expression of the insulin-dependent glucose transporter GLUT4 is abnormally
low. This has been shown in adipocytes from subjects with PCOS (67) as well
as in skeletal muscle from type 2 diabetics (68).

The mechanism of insulin resistance in the ovary also appears to involve a
post receptor abnormality leading to a selective signalling defect. The effect of
insulin on glucose metabolism in granulosa-lutein cells from women with
anovulatory PCOS is attenuated while the steroidogenic response to insulin is
preserved (71) (figure 1.11).
4.2 Aim

The overall aim of this project is to investigate the mechanism of insulin resistance in polycystic ovary syndrome

The specific aims were

In granulosa lutein cells from women with normal ovaries (control), those with endogenous androgen excess i.e women with polycystic ovary syndrome who continue to ovulate (ovPCO) and those who have irregular or anovulation (anov PCO)

1. to characterise the time and dose dependent effects of insulin action in glucose uptake and lactate accumulation in granulosa lutein cells in culture.

2. to assess insulin action on progesterone production

3. to assess insulin action on key phospho-proteins in the PI3K and MAPK signalling pathways
4.3 **Materials and Methods**

4.3.1 **Patients**

Granulosa cells were obtained, with informed, written consent, at the time of egg collection for IVF at Hammersmith Hospital from 31 women: 12 with normal ovaries, 8 with ovulatory PCOS (ov PCO) and 11 with anovulatory PCOS (anov PCO). These granulosa cells are isolated from the fluid that is collected from eggs in the ovary for IVF and are usually thrown away. Ethical approval for this project was sought from the Hammersmith Research Ethics Committee (REC) and obtained in Feb 2009 (reference number: 08/H0707/152)

Polycystic ovaries were defined using ultrasonography, by the presence of two or more of the following; 10 or more follicles 2-10mm in diameter, increased stromal density or volume, enlarged ovarian volume. Patients with polycystic ovaries and a history of irregular menstruation or amenorrhoea were classified as anov PCO. Those with a cycle length of between 21 and 35 days (but with no more than a 4 day variation between cycles) were classified as ov PCO. Clinical hyperandrogenism was defined as the presence of acne, hirsutism (Ferriman-Galway score ≥8) or alopecia. All 11 anov PCO patients and 6 of 8 ov PCO patients satisfied the Rotterdam criteria for diagnosis of PCOS (8).

The indications for IVF were as follows. In the normal ovary group, male infertility (n=7), tubal disease (n=2), unexplained (n=3). In the anov PCO group, anovulation resistant to ovulation induction (n=11), in addition there
was tubal disease in 1 case and male infertility in 4 cases. In the ov PCO group male infertility (n=6), unexplained (n=2).

4.3.2 Blood samples and follicular fluid aspiration

Peripheral venous blood for LH, FSH and oestradiol (E2) were taken between Day 3-6 of a pre-treatment assessment cycle from women with regular cycles, and at random in women with anov PCO who had oligo or amenorrhoea.

Insulin resistance was calculated from fasting plasma glucose and fasting serum insulin levels taken on the morning of the IVF procedure, by means of the Homeostatic Model Assessment (HOMA) algorithm (223). For this purpose, a mean average of two fasting glucose samples, obtained with a 30 minute collection interval, was used. Type 2 diabetes is associated with peripheral insulin resistance, elevated hepatic glucose production and inappropriate insulin secretion. The HOMA model is most useful for large scale population studies, but is fairly good as a description of individual insulin resistance as we have used here.

Testosterone (after extraction) and sex hormone binding globulin (SHBG) were also taken on the morning of the procedure and measured by immunoassay as previously described (48, 224) (Table 3).

Pituitary-gonadal suppression was achieved using GnRH analogue buserelin [(Suprecur, Sanofi-Aventis) 0.5ml sc daily for 1-3 weeks until gonadotrophin production is down-regulated]. Following this, superovulation of patients was achieved using recombinant FSH (Gonal-F, Serono). The anovulatory PCOS patients received the lowest doses of recombinant FSH, followed by the ovulatory PCOS patients in order to avoid ovarian hyperstimulation syndrome.
PCOS patients are at higher risk of developing this complication with IVF (table 3). Ultrasonography and serum E2 levels were used to monitor the patient’s response to FSH. When three or more follicles reached a diameter of 17mm and E2 levels reached 3000pmol/L, human chorionic gonadotrophin (hCG) 10000IU (Ovitrelle 250mcg. Serono) was administered. 32-36h after hCG administration, transvaginal follicle aspiration was performed. Oocytes were recovered and the follicular fluid aspirations from the left and right side were pooled and collected in sterile centrifuge tubes (TPP).

4.3.3 Human granulosa lutein cell collection, preparation and culture

![Flow diagram showing experimental protocol for human primary granulosa-lutein cell separation from red blood cells (RBCs), and preparation for experimentation. See text for description of percoll gradient preparation.](image)

**Figure 4.1**: Flow diagram showing experimental protocol for human primary granulosa-lutein cell separation from red blood cells (RBCs), and preparation for experimentation. See text for description of percoll gradient preparation.
All procedures were carried out under sterile conditions in a fume hood using aseptic tissue culture techniques. To enable removal of serum and reduce follicular fluid volume, samples were centrifuged at 200g for 5 minutes using a Centra CL3 (Thermo IEC) centrifuge. After discarding the clear follicular fluid supernatant, the pellets were then transferred into one 50mL centrifuge tube. If the volume of flush was too large, the pellets were centrifuged again and the process above repeated. The supernatant was then discarded and the cell solution volume was made up to 8mL with PBS.

In order to separate off any remaining red blood cells (RBCs) the follicular fluid mixture was gently layered over a percoll gradient. An autoclaved glass pipette was filled with cells and pipetted into 15mL centrifuge tubes containing 8mL pre-prepared shaken Percoll solution (8.9mL of Percoll [GE Healthcare], 1mL 10xM199, 100mL NaHCO₃ 7.5%, 10mL M199 [all from Invitrogen]). The 45% Percoll solution was filtered before use and shaken to produce bubbles, which provided a gentle surface for the cells to land on. The tubes were then centrifuged at 400g for 20-30min to isolate the granulosa-lutein cells; red blood cells separated off and sank to the bottom of the tube. If the cell mixture was seen to be particularly bloody a greater number of test tubes of percoll gradients were used.

Granulosa lutein cells were then aspirated from the Percoll/PBS interface with an autoclaved glass pipette and washed twice, using 10% serum supplemented M199. Cell number and viability were determined using a Trypan Blue exclusion test (Sigma). The experimental protocol is summarised in figure 4.1.
For the glucose, lactate and progesterone assays, 20 x 10⁴ cells were plated into 96 well plates, and supplemented with 200µl fetal bovine serum supplemented medium 199. Each experimental condition was repeated in five replicates. Cells were placed centrally and surrounding wells filled with sterile PBS to avoid evaporation. After 24h incubation at 37°C in 5% CO₂, serum containing medium was removed and the cells washed with PBS. They were then incubated for a further 24h with 200µL serum free Medium 199 with or without varying doses (1,10,100ng/ml) of Insulin (Sigma).

For the protein expression results, 50 x 10⁴ cells were added to 24 well plates and each well was supplemented with 1mL serum supplemented media. After 24h incubation at 37°C in 5% CO₂, the medium was removed and cells washed with PBS. They were then incubated for a further 24h with 1mL serum free Medium 199. The cells were the incubated with or without varying doses (10,100ng/ml) of Insulin (Sigma) for 5,10 or 15 minutes. There were only sufficient cells for one well per treatment.

Cells were then harvested in RIPA buffer, with aprotinin (MP Biochemicals, Cambridge, UK), protease inhibitors and sodium orthovanadate (Sigma). The samples were then centrifuged at 12000xg for 5 minutes at 4°C and supernatant collected. Laemmli sample suffer was added, and 20 µl loaded on a Bis-Tris NuPAGE pre cast polyacrylamide gel (Invitrogen). 5µl of a pre stained standard ladder was also loaded (Invitrogen) (figure 4.2).

### 4.3.4 Western Blotting

Gels were run using a wet-blotting system, X cell Surelock Mini-Cell system. The gels were then transferred using a semi-dry transfer system.
Polyvinylidene fluoride (PVDF) membrane (Thermo Scientific, p/a Perbio Science UK, Northumberland, UK), was cut to the size of the gel and soaked in ethanol or methanol for membrane activation. The transfer sandwich was then assembled in the following way: transfer sponge, followed by two sheets of Whatman chromatography paper, the PVDF membrane, the gel, a further two sheets of Whatman paper and finally another transfer sponge. The whole sandwich of layers was soaked in a dish filled with transfer buffer and a roller was rolled over the sandwich to remove trapped air. The apparatus was closed into the transfer machine and proteins were then transferred for 3.5h at 200mA.

4.3.5 Immunoblot analysis

The membrane was blocked with 5% skimmed milk and 1% 100mM sodium orthovanadate in 10% diluted 10x TBST buffer solution (Tris Buffered Saline with Tween) (blocking solution) for 15min at room temperature. Primary antibodies (appendix 1) were diluted in blocking solution and incubated with membranes overnight at 4°C. Blots were washed and incubated with near infrared (NIR) fluorophore labelled secondary antibodies were then used at a dilution of 1:10000 for 1 hour, prior to visualisation using the Odyssey infrared imaging system (LI-COR Biosciences).
Quantification of protein concentrations

ImageJ (Image processing and analysis in Java), was downloaded from the following website http://rsbweb.nih.gov/ij and was used to analyse the immunoblots. ImageJ and its Java source code are freely available and in the public domain. No license is required. It was developed at the Research Services Branch (RSB) of the National Institute of Mental Health (NIMH), part of the National Institutes of Health (NIH).

Each western jpeg file was dragged onto the ImageJ icon. The image was first converted to grayscale. The background was then subtracted. The next step involved selection of the measurements required, area, mean gray value, and
integrated density. Followed by selection of pixels as the unit of length required.

The colours on the image were then inverted, with the dark areas becoming light, and the light areas dark. As outlined above, this has the benefit of making the measured values for bands increase with increasing protein expression. Freehand selection was then chosen to draw a line around the boundary of the first band, using judgment about where the edges of the band are, and what is background noise. The enclosed area selected was then measured. The integrated density was calculated from the multiplication of the area by the mean gray value for each band. This was then repeated for each phosphorylation band and each control (GAPDH or total protein) and the results copied into an excel spreadsheet.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the key enzymes involved in glycolysis. It catalyzes the reversible oxidative phosphorylation of glyceraldehydes-3-phosphate. GAPDH is constitutively and stably expressed in almost all tissues at high level. It is a well-established "housekeeping" protein and is widely used as a loading control for protein normalization in western blotting (225). Insulin can stimulate GAPDH mRNA expression but this occurs over hours not minutes (226).

The integrated density of the phosphorylated protein band was then divided by the integrated density of the GAPDH or total protein band, to correct for any loading differences or cell/protein concentration differences between wells of the experiments.
It was then necessary to investigate whether the amount of phosphorylated protein was affected by the treatments administered i.e. insulin 10ng/ml or 100ng/ml. This was done by dividing the newly calculated loading protein concentration corrected integrated densities of the phosphorylated protein bands by the amount of phosphorylated protein seen in the control wells, unexposed to treatment for the same time points. Protocol summarised in figure 4.3.

![Flow chart detailing the protocol used to quantify amount of protein phosphorylation using image J software. (Integrated density=ID)](image)

**Figure 4.3:** Flow chart detailing the protocol used to quantify amount of protein phosphorylation using image J software. (Integrated density=ID)

### 4.3.7 Measurement of lactate production and glucose uptake

This has previously been described in Chapter 2, section 2.3.11. Individual 3-µl aliquots of culture medium from the experimental and control incubation
drops were collected using a 10µl pipette and diluted 1:200 for the glucose assay and 1:400 for the lactate assay, with a mixture of 5µM lactate and 2µM pyruvate solution. All samples were stored at -70°C until analysis with the COBAS bioanalyser.

4.3.8 Progesterone measurements

Progesterone levels in the spent medium were determined using the Abbott ARCHITECT i2000w automated Analyzer, which uses a chemiluminescent immunoassay. Individual 3-µl aliquots of culture medium from the experimental and control incubation drops were collected using a 10µl pipette and diluted 1:100 with serum free medium 199. Assays were performed in the Clinical Chemistry Laboratory, Hammersmith Hospital with the help of Clinical Biochemist Dr Mandy Donaldson.

4.3.9 Statistical analysis

Experiments to measure lactate, glucose and progesterone were done in five replicates. As there was considerable variability in basal values between subjects, the basal results of each patient prior to treatment was subtracted from results from each patient at 48h. The results of all the patients within a particular group (normal, ov PCO and anov PCO) were then combined and expressed graphically as mean +/- SEM. Statistical analysis was performed using Instat (Version 3 for Macintosh) and Prism software packages (Graphpad software, San Diego, CA, USA).
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<td><strong>BMI (kg/m^2)</strong></td>
<td>22.6 (1.1)</td>
<td>24.2 (1.2)</td>
<td>25.5 (1.6)</td>
<td>0.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LH (IU/L)</strong></td>
<td>4.6 (0.4)</td>
<td>4.8 (0.7)</td>
<td>11 (1.7)</td>
<td>0.0002</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>FSH (IU/L)</strong></td>
<td>6.8 (0.5)</td>
<td>4.7 (0.6)</td>
<td>5.2 (0.5)</td>
<td>0.02</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Fasting Insulin (mU/L)</strong></td>
<td>7.6 (0.84)</td>
<td>5.7 (1.2)</td>
<td>13 (2)</td>
<td>0.0042</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Fasting glucose (average, mmol/L)</strong></td>
<td>4.7 (0.1)</td>
<td>4.5 (0.1)</td>
<td>4.4 (0.1)</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>1 (0.1)</td>
<td>0.74 (0.2)</td>
<td>1.6 (0.2)</td>
<td>0.0049</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Testosterone (nmol/L)</strong></td>
<td>2.2 (0.2)</td>
<td>2.8 (0.2)</td>
<td>3.5 (0.5)</td>
<td>0.0475</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>SHBG (nmol/L)</strong></td>
<td>111.6 (9.2)</td>
<td>125.9 (17.4)</td>
<td>90.8 (15.3)</td>
<td>0.2308</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oestradiol pmol/L</strong></td>
<td>188.4 (14)</td>
<td>185 (11.6)</td>
<td>203 (17.6)</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FSH dose (IU)</strong></td>
<td>2406 (239.6)</td>
<td>1542 (144.5)</td>
<td>1235 (75)</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Ovarian volume (left side, ml)</strong></td>
<td>5.5 (0.3)</td>
<td>12.4 (1.2)</td>
<td>11.5 (1.2)</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Ovarian volume (right side, ml)</strong></td>
<td>6.3 (0.4)</td>
<td>10.8 (0.7)</td>
<td>12.1 (0.9)</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Follicle count (left)</strong></td>
<td>4.8 (0.6)</td>
<td>13.2 (1.2)</td>
<td>18.4 (2.4)</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Follicle count (right)</strong></td>
<td>4.7 (0.5)</td>
<td>12.9 (0.9)</td>
<td>18.4 (1.9)</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 3: Clinical and biochemical data [mean (SEM)] in normals, ov PCO and anov PCO patients. One way anova was used to perform statistical analysis between the groups.
4.4 Results

4.4.1 Patients recruited to the study

In total 66 patients were recruited. Of these, 10 were excluded because after processing and counting the cells there were insufficient numbers to plate in the wells. 4 developed fungal infections and were discarded. 11 patient samples were used in preliminary studies to achieve optimum plating density, washing technique, insulin and FSH treatment doses. Granulosa lutein (GL) cells from 2 controls were used in experiments to test GL response to insulin alone and insulin and androgen in combination (Chapter 2). 8 other patients were recruited but did not have complete data sets of information and as a result were unable to be included in the final analysis.

In total 12 controls, 8 ovulatory PCO patients and 11 anovulatory PCO patients were used in the study (Appendix 5).

4.4.2 Clinical and biochemical data of the patient subgroups

The clinical and biochemical data of the control, ovulatory PCOS and anovulatory PCOS patients recruited are summarised in Table 3 [arithmetic mean (SEM)].

The anovulatory PCOS group had a higher BMI [25.5 (1.2)] than both the control group [22.6 (1.1)] and ovulatory PCOS group [24.2 (1.2)], but this was not significant.

There were significantly higher levels of fasting insulin and HOMA-IR in the anovulatory PCO group than both the control and ovulatory PCOS group. Of note the ovulatory PCOS group were the most insulin sensitive with a HOMA-
IR 0.74 (0.2), control cells were the next most sensitive with a HOMA-IR of 1 (0.1), and the anovulatory PCOS group were least insulin sensitive with a HOMA IR of 1.6 (0.2). Type 2 diabetes is associated with peripheral insulin resistance, elevated hepatic glucose production and inappropriate insulin secretion. The HOMA model is most useful for large scale population studies, but is fairly good as a description of individual insulin resistance as we have used here.

Testosterone levels were highest in the anovulatory PCOS group [3.5nmol/L (0.5)], followed by the ovulatory PCOS group [2.8nmol/L (0.2)] and lowest in the control group [2.2nmol/L (0.2)].

Sex hormone binding globulin (SHBG) levels were found to be lowest in the anovulatory PCOS group [90.8nmol/L (15.3)] and highest in the control group [111.6nmol/L (9.1)] although this was not significantly different.

All 3 groups had oestradiol levels within the normal range [203pmol/L (17.3)] in the anovulatory group, [188.4pmol/L (14)] in the controls and [185pmol/L (11.6)] in the ovulatory PCOS group. There were no differences between the groups. As expected there were many more follicles in the anovulatory and ovulatory PCOS patients then in the control patients. Both the anovulatory PCOS group [1235IU (75)] and ovulatory PCOS group [1542 IU (144.5)] received significantly less FSH than the controls [2406IU (239.6)] during IVF for the reasons stated in section 4.3.2.

4.4.2 Glucose uptake, lactate and progesterone production

The data were analysed in different ways in order to find the best presentation. Although the same numbers of cells (20,000) were plated in
each well, over serial washing steps some cells were lost which may account for the differences in baseline glucose, lactate and progesterone values.

In order to address the variability of baseline glucose, lactate and progesterone values, we used the difference in glucose and lactate measurements in each well between time zero (the time when the cells were switched to serum free media) and 48h.

The response to insulin was most clear when changes in glucose uptake and lactate production and progesterone production were expressed as a percentage change from baseline (i.e no treatment).

**Figures 4.4-4.6**

Figures 4.4A, 4.5A, 4.6A shows the variable lactate production from (T0-T48h) and figure 4.4B, 4.5B and 4.6B, the variable glucose uptake, of individual anovulatory PCOS patients, control patients and ovulatory PCOS patients, in response to insulin 1-100ng/ml.

Each patient’s response is plotted in a separate colour. Beside each patient in figure 4.4A, 4.5A and 4.6A, data for BMI, HOMA IR, HOMA-S (measure of insulin sensitivity) and fasting insulin levels are listed to see if any correlation could be made between individual responses and their insulin sensitivity levels. No clear patterns were seen.
Figure 4.4: Control patients (n=12). (A) Lactate production of each patient from 0-48h (mmol/L), in response to no treatment or 1,10,100ng/ml insulin. Body mass index (BMI), insulin resistance (HOMA-IR), insulin sensitivity (HOMA-S) and fasting insulin (FI) of each patient have been listed by each patient. (B) Glucose uptake from the media of each individual patient from 0-48h (mmol/L), in response to 0,1,10,100ng/ml insulin.
Figure 4.5: Ovulatory PCOS patients (n=8). (A) Lactate production of each patient from 0-48h (mmol/L), in response to no treatment or 1,10,100ng/ml insulin. Body mass index (BMI), insulin resistance (HOMA-IR), insulin sensitivity (HOMA-S) and fasting insulin (FI) of each patient have been listed by each patient. (B) Glucose uptake from the media of each individual patient from 0-48h (mmol/L), in response to 0,1,10,100ng/ml insulin.
Figure 4.6: Anovulatory PCOS patients (n=11). (A) Lactate production of each patient from 0-48h (mmol/L), in response to no treatment or 1,10,100ng/ml insulin. Body mass index (BMI), insulin resistance (HOMA-IR), insulin sensitivity (HOMA-S) and fasting insulin (FI) of each patient have been listed by each patient. (B) Glucose uptake from the media of each individual patient from 0-48h (mmol/L), in response to 0, 1,10,100ng/ml insulin.
Percentage change from baseline for glucose uptake, lactate production and progesterone formation (figure 4.7)

The response to insulin was most clear when changes in glucose uptake and lactate production and progesterone production were expressed as a percentage change from baseline (i.e. no treatment).

The control group showed significantly higher lactate production than the anovulatory PCOS group (p=0.0235, Mann Whitney testing) in response to 10ng/ml insulin and an almost significantly higher response to 100ng/ml insulin (p=0.0694, Mann Whitney testing). The ovulatory PCOS group showed significantly higher glucose uptake than either the control or anovulatory PCOS group (p=0.0229, Kruskal Wallis testing) in response to 100ng/ml insulin.

Insulin stimulated progesterone production in a dose related manner in all three groups. No significant differences were seen between the groups.

The actual baseline values of glucose, lactate and progesterone are expressed as histograms in figure 4.8.
Figure 4.7: Percentage change: Glucose uptake, lactate production and progesterone production of granulosa lutein cells cultured in serum free culture for 48h. Calculated as difference of levels of glucose, lactate or progesterone in the wells from 0-48h, and expressed as percentage change from baseline. (A) Glucose uptake (Mean and SEM) in response to 1,10,100ng/ml insulin. (B) Lactate production (Mean and SEM) in response to 1,10,100ng/ml insulin. (C) Progesterone production (Mean and SEM) in response to 10,100ng/ml insulin.
Figure 4.8: Histograms: Glucose uptake, lactate production and progesterone production of granulosa lutein cells cultured in serum free culture for 48h. Calculated as difference of levels of glucose, lactate or progesterone in the wells from 0-48h (A) Glucose uptake (Mean and SEM) in response to no treatment, 10ng/ml and 100ng/ml insulin. (B) Lactate production (Mean and SEM) in response to no treatment, 10ng/ml and 100ng/ml insulin. (C) Progesterone production (Mean and SEM) in response to no treatment, 10ng/ml and 100ng/ml insulin.
4.4.3 Immunoblot quantification

Ser 473 phosphorylation of Akt

In 3 anovulatory PCO patients (SRo,LB,CN) 1 ovulatory PCO patient (LW) and 2 controls (LT and CD), it was very difficult assess total Akt levels using immunoblot analysis as the Akt immunostaining although present was too weak. For this reason data from these patients were not included in the final analysis of immunoblot results when Akt was used as the loading control. Figure 4.8 illustrates the variability of total Akt staining.

The GAPDH antibody however produced a strong response all the subjects and it was therefore possible to make a good assessment of protein loading in all subjects, thus no one was excluded.

Examples of blots from each subgroup of patients are illustrated in figure 4.9. The immunoblots illustrate the time dependent phosphorylation of Akt, ERK and GSK 3β phosphorylation to insulin 10,100ng/ml in all three groups of patients. Total Akt, ERK and GAPDH are also shown.
Control (ZD)

Ovulatory (EH)

Anovulatory (SF)

Figure 4.9: Examples of immunoblots from one patient in each group, control, ovulatory PCOS and anovulatory PCOS patients. Showing blots for phosphorylated Akt, phosphorylated ERK, total Akt, total ERK and GAPDH in response to insulin 10,100ng/ml and no treatment.
Dot plots and line graphs

The dot blots show each individual subject's response to insulin 10/100ng/ml at 5, 10 and 15 minutes, with a median line through the group of dots.

The line graphs show a mean and SEM for each patient group at 5, 10 and 15 minutes exposure to insulin 10/100ng/ml.

Akt phosphorylation using GAPDH as loading control (figure 4.10 dot plot and 4.11 line graph)

In response to 10ng/ml insulin, no significant changes were seen in any of the groups (using non parametric, kruskal-wallis testing, p=0.49).

In response to 100ng/ml insulin, no significant differences were seen between the groups (using non parametric, kruskal-wallis testing, p=0.24).
Figure 4.10: Western blot quantification of ser 473 phosphorylation of Akt using GAPDH as a loading control. Dot graph; Akt phosphorylation in response to insulin (0, 10, 100ng/ml) was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the fold change in expression for each individual patient, after 5, 10 or 15 minutes exposure to insulin, from baseline Akt phosphorylation in the presence of no treatment for each patient. Median values shown by horizontal line.
Western blot quantification of serine 473 phosphorylation of Akt using GAPDH as a loading control, line graph, line graph; Akt phosphorylation in response to insulin (0, 10, 100ng/ml) was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the average fold change in expression for all the patients in that group, after 5, 10 or 15 minutes exposure to insulin, from baseline Akt phosphorylation in the presence of no treatment for each patient group. Mean and SEM shown.

Figure 4.11: Western blot quantification of serine 473 phosphorylation of Akt using GAPDH as a loading control, line graph, line graph; Akt phosphorylation in response to insulin (0, 10, 100ng/ml) was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the average fold change in expression for all the patients in that group, after 5, 10 or 15 minutes exposure to insulin, from baseline Akt phosphorylation in the presence of no treatment for each patient group. Mean and SEM shown.
Akt phosphorylation, using total Akt as a loading control (figure 4.12 dot plot and 4.13 line graph)

In response to 10ng/ml insulin, no significant changes were seen in any of the groups (non parametric anova, kruskal-wallis p=0.44).

In response to 100ng/ml insulin, no significant changes between any of the groups. It did not make a difference whether analysis of Akt phosphorylation was done using total Akt or GAPDH as a loading control, both produced similar results (non parametric anova, kruskal-wallis, p=0.72)
Figure 4.12: Western blot quantification of serine 473 phosphorylation of Akt using Akt as a loading control, dot plot. Akt phosphorylation in response to insulin (0,10,100ng/ml) was quantified and expressed as a ratio of Akt expression (used as a loading control). Each dot on the graph represents the fold change in expression for each individual patient, after 5,10 or 15 minutes exposure to insulin, from baseline Akt phosphorylation in the presence of no treatment for each patient. Median values shown by horizontal line.
Figure 4.13: Western blot quantification of serine 473 phosphorylation of Akt using Akt as a loading control, line graph. Akt phosphorylation in response to insulin (0, 10, 100 ng/ml) was quantified and expressed as a ratio of Akt expression (used as a loading control). Each dot on the graph represents the average fold change in expression for all the patients in that group, after 5, 10 or 15 minutes exposure to insulin, from baseline Akt phosphorylation in the presence of no treatment for each patient group. Mean and SEM.
Serine 9 phosphorylation of GSKβ phosphorylation, using GAPDH as a loading control (figure 4.14 dot plot and 4.15 line graph)

No serine 9 or 21 phosphorylation was seen in 2 anovulatory PCO patients (CN, LB) and in 1 ovulatory PCO patient (LW). Therefore these patients were unable to be included in this analysis. The lower GSK 3β (ser 9) band was used for quantification, as it was clearer and easier to measure.

In response to 10ng/ml insulin, using Kruskal-Wallis testing no significant changes were seen between the groups in GSK 3β serine 9 phosphorylation (p=0.12)

In response to 100ng/ml insulin, no significant changes seen between any of the groups at any time points, using non parametric anova, kruskal-wallis testing, p=0.51.
Figure 4.14: Western blot quantification of serine 9 phosphorylation of GSK3β using GAPDH as a loading control, dot plot. GSK 3β phosphorylation (P-GSK 3β) in response to insulin (0, 10, 100ng/ml) was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the fold change in expression for each individual patient, after 5, 10 or 15 minutes exposure to insulin, from baseline GSK 3β phosphorylation in the presence of no treatment for each patient. Median values shown by horizontal line.
Figure 4.15: Western blot quantification of serine 9 phosphorylation of GSK3β using GAPDH as a loading control, line graph. GSK 3β phosphorylation (P-GSK 3β) in response to insulin (0,10,100ng/ml) was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the average fold change in expression for all the patients in that group, after 5,10 or 15 minutes exposure to insulin, from baseline GSK 3β phosphorylation in the presence of no treatment for each patient group. Mean and SEM.
Phosphorylation of ERK 1/2 p44/42. The p42 band (Erk 2) was used for quantification, as this appeared clearer on all the membranes.

In 2 anovulatory PCO patients (LB,CN) 1 ovulatory PCO patient (LW) and 2 controls (LT and AK), it was very difficult assess ERK levels using immunoblot analysis as the ERK immunostaining although present was very weak. For this reason this patients were not included in the final immunoblot results when ERK was used as a loading control. The GAPDH antibody however picked up protein levels in all the subjects and it was therefore possible to make a good assessment of protein loading in all subjects, thus no one was excluded.

Measurement of ERK phosphorylation using GAPDH as a loading control (figure 4.16 dot plot, figure 4.17 line graph)

In response to 10ng/ml insulin, no significant changes were seen between any of the groups.

In response to 100ng/ml insulin, there was a statistically significant difference between the groups at both 10 minutes (p=0.0088) and 15 minutes (p=0.03). Statistics done using non parametric testing, Kruskal-Wallis, as not all data was normally distributed.

Specifically there was a significantly higher phosphorylation of ERK in the control group compared to the ov PCO group at both 10 minutes (p=<0.01) and 15 minutes (p=<0.05).
Figure 4.16: Western blot quantification of p42 phosphorylation of ERK using GAPDH as a loading control, dot plot. ERK phosphorylation in response to insulin (0, 10, 100ng/ml) was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the fold change in expression for each individual patient, after 5, 10 or 15 minutes exposure to insulin, from baseline ERK phosphorylation in the presence of no treatment for each patient. Median values shown by horizontal line.
Figure 4.17: Western blot quantification of p42 phosphorylation of ERK using GAPDH as a loading control, line graph. ERK phosphorylation in response to insulin (0, 10, 100ng/ml) was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the average fold change in expression for all the patients in that group, after 5, 10 or 15 minutes exposure to insulin, from baseline ERK phosphorylation in the presence of no treatment for each patient group. Mean and SEM.
Using ERK p44/22 as a loading control (figure 4.18 dot plot, figure 4.19 line graph)

Similar results are seen when using ERK as a loading control to those seen with GAPDH as a loading control. In response to 10ng/ml insulin, no significant changes seen between any of the groups.

In response to 100ng/ml insulin, there was a significantly higher phosphorylation of ERK in the ov PCOS group compared to controls at 10 minutes (p=0.028). No other significant changes were seen. Statistics done using kruskal-wallis, non parametric testing.
Figure 4.18: Western blot quantification of p42 phosphorylation of ERK using ERK as a loading control, dot plot. ERK phosphorylation in response to insulin (0, 10, 100ng/ml) was quantified and expressed as a ratio of ERK expression (used as a loading control). Each dot on the graph represents the fold change in expression for each individual patient, after 5, 10 or 15 minutes exposure to insulin, from baseline ERK phosphorylation in the presence of no treatment for each patient. Median values shown by horizontal line.
Figure 4.19: Western blot quantification of p42 phosphorylation of ERK using ERK α loading control, line graph. ERK phosphorylation in response to insulin (0, 10, 100 ng/ml) was quantified and expressed as a ratio of ERK expression (used as a loading control). Each dot on the graph represents the average fold change in expression for all the patients in that group, after 5, 10 or 15 minutes exposure to insulin, from baseline ERK phosphorylation in the presence of no treatment for each patient group. Mean and SEM.
4.4.4 Summary of results

There is evidence for insulin dependent changes in glucose metabolism and progesterone production in granulosa-lutein cells. Women with anovulatory PCOS have impaired glucose metabolism with reduced glucose uptake and lactate production with preserved insulin stimulated progesterone formation, as previously shown by Rice et al (71).

There is evidence for insulin-mediated activation of PI3K and MAPK signalling pathways. No differences were seen between the groups in PI3K signalling but there was a reduction of ERK phosphorylation in the ovulatory PCOS group compared to controls.

No impairment of the insulin stimulated PI3K was seen between the women with PCOS and without PCOS, using 2 signalling proteins, serine 473 phosphorylation of Akt and serine 9 phosphorylation of GSK 3β phosphorylation. No phosphorylation of FOXO 01/03 was seen in any of the groups of patients (data not shown).

No obvious pattern in PI3K signalling or glucose and lactate metabolism can be seen when looking directly at the insulin sensitivity of individuals.
4.5 Discussion

We confirmed the selective resistance to insulin in glucose metabolism in GL cells in women with anovulatory PCOS, with preservation of gonadotrophin-stimulated glucose uptake and metabolism seen by Rice et al. (71).

It was hypothesized that when comparing PI3K signalling in GL cells from women with anovulatory PCOS who are the most insulin resistant group and had significantly higher testosterone levels, than controls, that there would be a significant difference in Akt phosphorylation between the groups. This was not shown to be the case. No significant differences were seen in Akt or GSK 3β phosphorylation between the groups. This remained the case whether GAPDH or total Akt protein was used as a loading control. There was however a time dependent response in Akt and GSK3β phosphorylation to insulin at both doses in all groups. The Akt and GSK3β phosphorylation ratios of each patient were also analysed alongside their individual HOMA IR levels and no obvious correlations were seen (data not shown).

The absence of any obvious impairment in the insulin-stimulated PI3K signalling pathway in response to insulin in women with anovulatory PCOS is unexpected. We did however see significant impairment in MAPK signalling between women with ov PCO and control patients, the significance of this finding with respect to impaired glucose metabolism in granulosa cells remains to be determined. Again this remained the case whether GAPDH or total ERK was used as a loading control. All three patient groups made similar amounts of progesterone and there were no significant differences seen.

The reduction in expression of p42-44 ERK in women with ovulatory PCOS
compared to controls is interesting. There is some data to suggest a role for p38 MAPK in glucose metabolism in cardiac myocytes, 3T3-L1 adipocytes, and skeletal muscle.

In vivo differentiation of cardiac myocytes involves, up-regulation of GLUT4 and down regulation of GLUT1 (227-229). Studies in primary cardiac myocytes have shown that in culture, primary adult cardiomyocytes de-differentiate into a fetal program of GLUT gene expression, up-regulating GLUT1 and down-regulating GLUT4. Treatment with IGF-1 restored expression of GLUT4 protein and mRNA, and this was mediated by the p38 MAPK axis. Use of a p38 inhibitor SB203580, completely abolished the overexpression of GLUT4 induced by 500ng/ml IGF-1. Transient transfection experiments in neonatal cardiac myocytes confirmed p38 MAPK can activate the GLUT4 promoter. Transient transfection of the ERK1/2 mutant DN3MEK1 did not significantly alter GLUT4 promoter activity, whereas p38 MEK6DD elicited good stimulation of GLUT4 promoter activity, which was entirely blocked by SB203580 (230).

Another study this time in 3T3-L1 adipocytes showed that sodium arsenite (a protein modifying agent) stimulates glucose uptake in 3T3-L1 adipocytes (231-233). Using western blotting this was shown to be mostly via translocation of GLUT4 and GLUT1 to the cell surface. Arsenite in this study did not activate other signalling steps normally activated in response to insulin such as IR tyrosine kinase, IRS-1 and IRS-2 tyrosine phosphorylation, Akt phosphorylation or PKC-λ, suggesting that the target of arsenite is downstream of PI3K or in a separate pathway. Both arsenite and insulin induced glucose uptake were partially inhibited by p38 MAPK inhibitor
Work in the L6 muscle cell line has shown that insulin stimulated GLUT4 translocation precedes the stimulation of glucose uptake in time, suggesting that GLUT4 may undergo an activation step following its insertion into the plasma membrane (234). Using p38 MAPK inhibitors SB203580 and a dominant negative p38 MAPK mutant, it has been shown that insulin stimulated glucose uptake is attenuated without altering GLUT4 translocation, suggesting insulin may activate GLUT4 via a p38 MAPK pathway (235, 236).

The studies above have been done looking at the effect of p38 MAPK in glucose metabolism and our study looked at the effect of p42/44 ERK. Little conclusive evidence has been found for an effect of p42/44 ERK on glucose metabolism. MAPK is capable of phosphorylating an impressive array of target proteins in vitro, many of which have a regulatory role and are known to be phosphorylated in response to insulin. For example, GSK-3α and β are phosphorylated and inactivated by two protein kinases ribosomal protein S6, the 90kDa S6 (also known as 90rsk or MAPKAP-K1) (79) and 70KDa S6 which lie downstream of MAPK (80, 81). But this only gives us circumstantial evidence in favour of a role for MAPK in insulin action. Studies ablating MAPK activity using truly specific inhibitors or mRNA targeting, have been hampered by the presence of multiple related MAPK isoforms (82). It would be useful to investigate the expression of p38 MAPK in our system. Interestingly p42/44 MAPK signalling in primary human thecal cells from women with PCOS has been shown to be by down-regulated, and this was associated with an increase in androgen production (237).
It is possible that there may be defects in pathways other than PI3K and MAPK that we have not investigated for, such as the PKC, AMPK or FOXO pathways. AMPK is an intracellular energy sensor that plays a central role in glucose and lipid metabolism. AMPK activates when cellular energy is depleted and accelerates ATP generating catabolic pathways such as oxidation of fatty acid and glucose (238-240), and at the same time reduces ATP consuming anabolic pathways such as fatty acid synthesis (241, 242). Activators of AMPK enhance glucose uptake in 3T3-L1 adipocytes. Using GFP labelled GLUT4 and live confocal imaging, it has been shown that AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), accelerates GLUT4 translocation within 4 minutes. GLUT4 translocation is not affected by wortmannin (PI3K inhibitor), but wortmannin did inhibit AICAR and insulin induced glucose uptake. SB-203580 an inhibitor of p38 MAPK also did not inhibit GLUT4 translocation but did prevent the enhancement of glucose uptake induced by AMPK and insulin. It is possible that the increase in glucose uptake seen with AMPK activators may be via a p38 MAPK dependent mechanism (243).

So far, 12 PKC isoforms have been cloned in mammals, consisting of PKC three subgroups: classical or conventional PKCs (α, βI, βII and γ), novel PKCs (PKCδ, ε, η and θ), and atypical PKC (PKCζ, λ). There is evidence to indicate that atypical PKCs (aPKCs) are involved in the regulation of glucose metabolism and play a crucial role in pathogenesis in type 2 diabetes (244). Atypical PKCs are thought to have a role in induction of insulin mediated GLUT4 translocation to the plasma membrane. There are 2 distinct pathways
that are thought to be involved in glucose transport, PI3K and the Cbl-TC10 pathway, aPKCs function as a downstream target of both (245).

The second pathway Cbl-TC10, involves insulin binding to the insulin receptor and activating protein Casitas B lineage Lymphoma (Cbl). Which is recruited to the insulin receptor via interaction with adapter proteins CAP and APS. On phosphorylation the Cbl-CAP complex dissociates from the insulin receptor and moves to the caveolin rich membrane fraction. The complex then binds a protein flotillin and this ternary complex is recruited to lipid rafts in the plasma membrane (246, 247). TC10 a member of the Rho family is constitutively localized to plasma membrane lipid raft domains. Activated Cbl recruits CRKII-C3G complex here to lipid rafts, where C3G specifically activates TC10. TC10 then activates aPKCs through a Par 6-3 interaction (245, 248) (figure 4.20).

Figure 4.20: Atypical protein kinase in insulin sensitive cells, from Liu et al. (244)
One study done in adipocytes correlates well with our work and supports the idea of a defect in the atypical PKC pathway. The action of androgen on pre-adipocytes from healthy women treated with testosterone or anti-androgens for 48h was investigated. Maximal insulin stimulated glucose uptake (insulin 10nM) was impaired in the cells treated with testosterone 10nmol/L and 100nmol/L. This defect was attenuated by co-incubation with anti-androgens. However testosterone treatment did not alter phosphorylation of insulin receptor substrate-1, Akt or MAPK. It did however impair atypical protein kinase C (PKC) isoform ζ phosphorylation (188). PKC as described above is activated by PI3K and PDK, and has been shown to be important in glucose metabolism (204).

However some studies in adipocytes have shown defects in the PI3K pathway. A study done in adipocytes from women with polycystic ovary syndrome demonstrated reduced insulin stimulated glucose uptake in adipocytes from women with PCOS compared to controls. The PCOS adipocytes showed higher tyrosine phosphorylation and lower serine phosphorylation of GSK 3β, indicating a higher GSK3β activity and therefore reducing glycogen synthase activity. The GSK 3β overactivity was confirmed using a radioactive GSK 3β activity assay (249).

Akt also phosphorylates and inactivates members of the forkhead transcription factor family (FOXO) by causing its nuclear exclusion and inactivation (89). FOXO transcription factors play an important role in modulating metabolic functions. Given relatively high expression of FOXO1 in insulin-responsive tissues and the impact of insulin signalling on Akt-mediated phosphorylation of FOXO, this transcription factor is highly poised to regulate
energy metabolism. When insulin and nutrient levels are low, FOXO1 promotes expression of gluconeogenesis enzymes. In insulin resistance, negative signalling to FOXO1 is compromised (250). However in our study we failed to find any evidence of FOXO1 or FOXO3 phosphorylation (data not shown). FOXO 01, FOXO 03 and FOXO 04 have all been seen previously in human mural luteinized granulosa cells using western blotting (251). Due to time and sample limitations we did not run blots for total FOXO1.

Another suggestion is that the signalling defect in the PI3K pathway involves a protein in the signalling pathway that is more proximal to Akt, such as IRS1 or the insulin receptor itself.

Primary differentiated rat skeletal muscle myotubes were subjected to 30min insulin 100nM and showed serine phosphorylation of IRS-1 \(^{636/639}\) and Akt \(^{473}\) were increased. When these cells were subjected to 16h pre-exposure to testosterone (20ng/ml), they showed a significant increase in insulin induced serine phosphorylation of IRS-1 \(^{636/639}\) (p=0.042), insulin induced m-Tor-ser\(^{2448}\) and insulin induced S6K-Thr\(^{389}\) were increased, compared to cells not pre-exposed to testosterone (252). Higher dose of testosterone 200ng/ml showed a non significant rise in insulin induced serine phosphorylation of IRS-1 \(^{636/639}\) (p=0.2).

Another protein of interest is IRS-1 ser\(^{312}\) phosphorylation. Cultures of skeletal muscle in obese women with PCOS have been studied. Basal and insulin stimulated GLUT1 abundance were significantly increased. IRS-1 protein abundance was significantly increased in PCOS resulting in significantly decreased PI3K activity when normalised for IRS-1. Phosphorylation of IRS-1 ser\(^{312}\) (equivalent to Ser\(^{307}\) in rat) was significantly increased in PCOS, but
despite these defects the cultured myotubules showed normal insulin responsiveness. No differences in IR tyrosine phosphorylation were seen between women with PCO and controls. In contrast to skin fibroblasts the defects in skeletal muscle seen in vivo resolve in culture consistent with the major role of the in vivo environment in insulin resistance. However the defects in insulin signalling persist (127). Serine phosphorylation of IRS1 inhibits its tyrosine phosphorylation and therefore its ability to recruit PI3K (62).

IR serine phosphorylation is another area of possible differences. Isolated skin fibroblasts of about 50% of women with PCOS who had insulin resistance documented by euglycaemic clamp studies, have constitutively active serine phosphorylation of the insulin receptor that inhibits insulin-stimulated tyrosine phosphorylation (130).

This was also shown in a study using primary human granulosa cells from women with insulin resistant PCOS (IR-PCOS) and non insulin resistant PCOS (NIR-PCOS), measured by continuous infusion of glucose model assessment test (CIGMA), as well as controls. Cells cultured in vitro with insulin and/or LH showed that insulin stimulated lactate production in the NIR-PCOS and control group but not in the IR-PCOS group. This was associated with a down regulation of insulin receptor (assessed using western blotting) in the IR-PCOS group (253).

Conclusions

The selective resistance to insulin in glucose metabolism in GL cells in women with anovulatory PCOS was confirmed. The absence of any obvious
impairment in the insulin-stimulated PI3K signalling pathway in response to insulin in women with anovulatory PCOS is unexpected. We did however see impairment in MAPK signalling and the significance of this finding with respect to impaired glucose metabolism in granulosa cells remains to be determined.

It may be possible that granulosa cells are behaving in a similar way to brain and red blood cells, where insulin action is far less important in glucose metabolism than in tissues such as the liver and skeletal muscle. There is evidence that gonadotrophins are involved in glucose metabolism in granulosa cells (70, 71) (Chapter 5), and may well be the primary regulator of glucose metabolism in these cells.
Chapter 5

Effects of FSH on glucose metabolic pathways in human primary granulosa lutein cells
5.1 Introduction

FSH has been shown to have important effects on glucose metabolism. FSH significantly increased oocyte maturation and produced a 2-3 fold increase in glucose uptake and lactate production by cumulus oocyte complexes in which the enclosed oocyte completed maturation (70). In this study, the PI3-kinase inhibitor LY294002 inhibited FSH stimulated glucose uptake, and GLUT4 glucose transporter was found in granulosa cells suggesting that FSH increased glucose uptake by PI3-kinase-mediated translocation of GLUT4 to the granulosa cell membrane (70).

Gonadotrophins are thought to be involved in energy metabolism during follicle development. Hillier et al. showed granulosa cells respond to gonadotrophins by increasing lactate production (95).

Rice et al. used granulosa-lutein cells from women with anovulatory PCOS, ovulatory PCOS, and a control group of women and looked into the effects of insulin (1-1000ng/ml) and LH (1-5ng/ml) on carbohydrate metabolism and progesterone production. Cells from the anovulatory PCOS subjects were significantly less responsive to insulin in lactate production than those from both of the other two groups. The addition of LH however resulted in a dose-dependent increase in glucose uptake, which was similar in each of the groups as well as dose-dependently, stimulated lactate and progesterone production in each group (figure 1.12). Therefore in contrast to the response to insulin, there was no significant difference in response to LH between the groups. LH stimulated progesterone production in all three groups with a peak response of two to three times that of the basal value. Progesterone production by the anovulatory PCOS cells was significantly greater than from
the control (P=0.046, ANOVA) and ovulatory PCOS (P=0.03, ANOVA) cells (71).

Several studies have shown an effect of FSH on steroidogenesis in human primary granulosa lutein cells taken from normal women undergoing IVF (190-193). However this is the first study in primary human granulosa lutein cells to look at gonadotrophin effects on downstream signalling pathways involved in glucose metabolism. Previous work in luteinized GL cells has looked at the effect of LH but not of FSH.
5.2 Aim

The purpose of this project is to assess the effect of FSH on human primary granulosa lutein cells [that are harvested at the time of egg collection for in vitro fertilization (IVF)] on glucose metabolism and downstream signalling pathways involved in glucose metabolism.

Specific aims were:

1. to assess FSH action on glucose metabolism
2. to assess FSH action on progesterone production
3. to assess FSH action on PI3K and MAPK signalling pathways

in granulosa lutein cells from women with normal ovaries and those with endogenous androgen excess i.e women with polycystic ovary syndrome.
5.3 Materials and Methods

Details of the materials and methods used in the chapter are given in Chapter 4. In brief, patient groups are described in section 4.3.1 and blood samples and follicular fluid aspirations are described in section 4.3.2. Human granulosa lutein cell collection, preparation and culture are described in section 4.3.3.

For the glucose, lactate and progesterone assays, $20 \times 10^4$ cells were plated into 96 well plates, and supplemented with 200µl serum supplemented medium 199. Each experimental condition was repeated in five replicates. Cells were placed centrally and surrounding wells filled with sterile PBS to avoid evaporation. After 24h incubation at $37^\circ\text{C}$ in 5% CO$_2$ serum containing medium was removed and the cells washed with PBS. They were then incubated for a further 48h with 200µL serum free Medium 199 with or without FSH 20ng/ml (dissolved in PBS +0.1% bovine serum albumin, National Hormone and Pituitary Program, California, USA). FSH 20ng/ml was chosen as it is a physiological dose.

For the protein expression results, $50 \times 10^4$ cells were added to 24 well plates and each well was supplemented with 1mL serum supplemented media. After 24h incubation at $37^\circ\text{C}$ in 5% CO$_2$, the medium was removed and cells washed with PBS. They were then incubated for a further 24h with 1mL serum free Medium 199. The cells were the incubated with or without FSH 20ng/ml (dissolved in PBS +0.1% bovine serum albumin, appendix 3) for 5,10 or 15 minutes. Cells were then harvested in RIPA buffer, with aprotinin (MP Biochemicals, Cambridge, UK), protease inhibitors and sodium orthovanadate
(Sigma). The samples were then centrifuged at 12000x g for 5 minutes at 4°C and supernatant collected. Laemmli sample suffer was added, and 20 µl loaded on a Bis-Tris NuPAGE pre cast polyacrylamide gel (Invitrogen). 5µl of a pre stained standard ladder was also loaded (Invitrogen) (figure 4.2).

Western blotting technique is described in section 4.3.4 and immunoblot analysis described in section 4.3.5. Protein concentration quantification is described in 4.3.5. Glucose, lactate and progesterone measurement are described in section 4.3.6 and 4.3.7. Finally statistical analysis is described in section 4.3.8.
5.4 Results

5.4.1 Glucose uptake, lactate and progesterone production in response to FSH 20ng/ml

As previously described in section 4.4.2, in order to address the variability of baseline glucose and lactate and progesterone values, we used the difference in glucose and lactate measurements in each well between time zero (the time when the cells were switched to serum free media) and 48h. The response to FSH 20ng/ml was expressed as a percentage change from baseline (figure 5.1).

Effects of FSH on glucose metabolism

FSH 20ng/ml induced glucose uptake from the media and lactate production in all 3 groups of patients, controls, ovulatory PCOS and anovulatory PCOS. The FSH induced rise in lactate production was statistically significant in both the anovulatory PCOS (p=0.0004, Mann Whitney) and ovulatory PCOS groups (p=0.023), but not in the control group (p=0.45).

The effect of FSH on glucose uptake was similar to that of lactate. Compared to no treatment, FSH 20ng/ml produced a significant rise in glucose uptake in both the anovulatory PCOS (p=0.01, Mann Whitney) and ovulatory PCOS groups (p=0.02), but not in the control group (p=0.3).

Compared to no treatment, FSH 20ng/ml produced a significant rise in progesterone production in all three groups, anovulatory PCOS (p<0.0001, Mann Whitney), ovulatory PCOS (p<0.0001, Mann Whitney), and controls (p<0.0001, Mann Whitney).
Comparison of the FSH response between the three groups was made using non parametric ANOVA (Kruskal-Wallis). There was a significant difference in lactate production between the 3 groups (p=<0.0001). There was a significantly higher lactate production from the anov PCO group compared to both the controls, and ov PCO group. There was also a significant difference in glucose uptake between the 3 groups (p=0.02). There was also a significantly higher glucose uptake from the anov PCO group compared to the controls.

FSH 20ng/ml induced a highly significant (3-4 fold) increase in progesterone production in GL cells in all three groups of subjects (p<0.0001 in each group; Mann Whitney). However, there was no significant difference in progesterone production between the three groups (figure 5.1).

The absolute values for glucose uptake, lactate and progesterone production are depicted as histograms in figure 5.2.
Figure 5.1: Glucose uptake, lactate production and progesterone production of primary granulosa lutein cells cultured in serum free culture for 48h. Calculated as difference of levels of glucose, lactate or progesterone in the wells from 0-48h, and expressed as percentage change from baseline. (A) Glucose uptake (Mean and SEM) in response to 20ng/ml FSH (B) Lactate production (Mean and SEM) in response to 20ng/ml FSH (C) Progesterone production (Mean and SEM) in response to 20ng/ml FSH.
Chapter 5

Figure 5.2: Histograms, Glucose uptake, lactate and progesterone production of primary granulosa lutein cells cultured for 48h in response to FSH 20ng/ml. (A) Glucose uptake (absolute values before and after treatment) in response to 20ng/ml FSH (B) Lactate production (absolute values before and after treatment) in response to 20ng/ml FSH (C) Progesterone production (absolute values before and after treatment) in response to 20ng/ml FSH.
5.4.2 FSH signalling in primary granulosa lutein cells

Examples of blots from each subgroup of patients are illustrated in figure 5.3. The immunoblots illustrate the time dependent phosphorylation of Akt, ERK and GSK 3β phosphorylation in response to FSH 20ng/ml in all three groups of patients. Total Akt, total ERK and GAPDH are also shown.

FSH 20ng/ml produced very little PI3K activation as seen in figure 5.1. Akt phosphorylation was unaffected by the addition of FSH. There was also no GSK3β phosphorylation in response to FSH 20ng/ml in any of the groups. It was therefore not possible to quantify the low amount of Akt or GSK3β phosphorylation in response to FSH 20ng/ml.

ERK phosphorylation was however detectable in response to FSH 20ng/ml. This is seen clearly in the control and anovulatory PCOS patient examples below. There was as expected variation from subject to subject; so for example little evidence of ERK phosphorylation is seen in the particular ovulatory PCOS subject shown here.
Figure 5.3: Examples of immunoblots from one patient in each group, control, ovulatory PCOS and anovulatory PCOS patients. Shown are blots for phosphorylated Akt, phosphorylated ERK, total Akt, total ERK and GAPDH in response to FSH 20ng/ml and no treatment.
5.4.3 Quantification of phospho-proteins

As in Chapter 4, when measuring phosphorylation of ERK 1/2 p44/42, the p42 band (ERK 2) was used for quantification, as this appeared clearer on all the membranes.

ERK phosphorylation was quantified using GAPDH as a loading control (dot plot shown in figure 5.4, and line graph in figure 5.5). The dot plots show each individual subjects response to FSH 20ng/mL at 5, 10 and 15 minutes. The line graphs show a mean and SEM for each patient group at 5, 10 and 15 minutes exposure to FSH.

In response to 20ng/ml FSH, no significant changes were seen between any of the groups at any time point. Statistics were done using the Kruskal-Wallis test, as not all data were normally distributed.
Figure 5.4: Western blot quantification of p42 phosphorylation of ERK using GAPDH a loading control, dot plot. ERK phosphorylation in response to FSH 20ng/ml was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the fold change in expression for each individual patient, after 5, 10 or 15 minutes exposure to FSH 20ng/ml, from baseline ERK phosphorylation in the presence of no treatment for each patient. Median values shown by horizontal line.
Figure 5.5: Western blot quantification of p42 ERK phosphorylation using GAPDH as a loading control, line graph. ERK phosphorylation in response to FSH 20ng/ml was quantified and expressed as a ratio of GAPDH expression (used as a loading control). Each dot on the graph represents the average fold change in expression for all patients in that group, after 5, 10 or 15 minutes exposure to FSH 20ng/ml, from baseline ERK phosphorylation in the presence of no treatment for each patient group. Mean and SEM shown.
5.5 Discussion

FSH 20ng/ml stimulated significant glucose uptake and lactate production in both the anov PCO and ov PCO groups although not in GL cells from the control subjects with normal ovaries. The variant responses are not easy to explain but could be related to the difference in the number of FSH receptors (FSHR) expressed in the GL cells of anov PCO patients compared to controls. The response to FSH in patients undergoing IVF has been shown to be positively correlated to the expression level of FSHR in granulosa-lutein cells (figure 5.6). Compared with patients with no ovarian factor of infertility, the FSHR expression level is significantly higher in patients with PCOS (254-256). This may also explain why there was a significantly greater lactate production and glucose uptake in the anovulatory PCO patients in response to FSH 20ng/ml compared to the control subjects.

![Figure 5.6](image)

**Figure 5.6**: Variable FSHR expression in IVF patients. Mean and SD of expression levels (relative to β-actin, using qPCR) of FSHR, in patients with different infertility diagnoses. NOF = no ovarian factor; PR = poor responders; EM-A = endometriosis with ≤10 oocytes retrieved; EM-B = endometriosis with >10 oocytes retrieved; PCOS = polycystic ovary syndrome. For each bar graph, the results of comparisons between groups are reported as follows: a = significantly different from NOF; b = significantly different from poor responders; c = significantly different from endometriosis-A; d = significantly different from endometriosis-B; e = significantly different from PCOS. From Gonzalez-Fernandez et al. 2011(256)
One explanation for the higher FSHR levels seen in PCOS GL cells is hyperandrogenism. Using quantitative RT-PCR, AR gene expression has been reported in GL cells from human stimulated preovulatory follicles. A higher expression of AR was seen in smaller follicles than larger follicles in PCOS (255). Increased AR expression in GL cells of testosterone-treated monkeys from the pre-antral to the large antral follicle stage has also been shown; and primate ovary AR gene expression is most abundant in granulosa cells of healthy growing follicles, where its expression is up-regulated by testosterone. These data support the view that androgens stimulate AR expression (257). Both AR and FSHR expression were strongly and positively correlated in small follicles from PCOS patients and controls and in large follicles from PCOS patients (255). Weil et al. (258) also reported a highly significant positive correlation between FSHR and AR mRNA levels in GL cells from testosterone-treated or control primate follicles. Androgen treatment significantly increased FSHR mRNAs in GL cells (by ~50–100%, depending on follicle size) (258).

The greater number of FSH receptors in patients with PCOS also explains the requirement of lower doses of exogenous recombinant FSH during IVF in order to prevent ovarian hyperstimulation (Table 3).

The differences between the groups in the response of glucose and lactate to FSH is in surprising contrast to the changes induced by insulin in GL cells (see chapter 4). Furthermore, in our study unlike the effect of insulin, FSH 20ng/ml did not show any measurable levels of Akt and GSK 3β phosphorylation. FSH 20ng/ml did however show measureable increases in p42/44ERK phosphorylation, which may suggest an involvement of the MAPK
pathway in glucose metabolism or may simply reflect changes in the pathways involved in FSH-induced progesterone production. Another possibility is that an alternative signalling pathway such as AMPK or atypical PKC is involved in FSH induced glucose metabolism but this has not yet been investigated in our system.

ERK was phosphorylated in a time dependent manner for all three groups of subjects however there were no significant differences seen between the groups. Alongside this result, FSH 20ng/ml produced a significant rise in progesterone production compared to no treatment in all three groups.

In conclusion, in primary human granulosa lutein cells FSH 20ng/ml has an effect on MAPK signalling but little effect on PI3K signalling. Compared to no treatment addition of FSH 20ng/ml produced significant glucose metabolism in ovPCO and anovPCO subjects, but not in controls. This is the first study to investigate the effect of FSH on glucose metabolism in primary human granulosa cells. We conclude that, as in the mouse ovary, FSH can induce glucose uptake and lactate accumulation but without clear evidence of activation of the PI3-kinase pathway.
Conclusion
Conclusion

KK1 cells were useful for looking into the action of insulin and androgen as well as the positive interactive effect of insulin and androgen. This could be said to be a quirk of cell line work, however the fact that it was possible to repeat these experiments in primary human granulosa lutein cells from two control patients, suggests otherwise. Rather than the expected inhibition, of insulin-stimulated glucose uptake and lactate production, this augmentation by androgens is a surprising result.

The results suggest that the insulin resistance observed in granulosa cells from women with PCOS, cannot be explained by direct exposure to androgen. Perhaps other circulating factors in vivo such as fatty acids or adipokines produced by fat cells such as tumour necrosis factor α (TNF-α), leptin and adiponectin are contributing to insulin resistance in granulosa cells.

Future studies could employ techniques such as microarray analyses to identify which genes are up regulated by androgen therapy in KK1 cells. One anomaly noted was that glucose uptake and lactate production, were only detectable after 24h of exposure to substrates such as insulin, but phosphorylation of Akt and ERK and also movement of GLUT4 were seen within minutes. It is possible that the amount of glucose uptake was simply too small to be detected any earlier by the COBAS bio-analyser, and if time had permitted an alternative method to measure glucose uptake such as uptake of radio-labelled glucose from the medium would have been a more sensitive method of monitoring small amounts of glucose taken up rapidly by KK1 cells.

The KK1 cells were less useful for looking into the action of FSH although this was not our primary objective, and they were also not useful in investigating the action of hormones on steroidogenesis. This could be because the Abbott
analyser, which is used clinically in the biochemistry department of Hammersmith hospital to measure progesterone concentrations in the blood in nanomolar concentrations, was not sensitive enough to detect the amounts produced by the KK1 cells. It may also be explained by inadequate transfection of KK1 cells with the FSH receptor. It is also possible that phenotypic drift of the cell line over repeated passages may have had an effect. It would be useful to look for gene expression of FSH sensitive genes, or immunocytochemistry for the FSH receptor.

The results of the human primary granulosa lutein cell experiments confirmed selective impairment of glucose metabolism in cells from anovulatory PCOS. It was hypothesized that when comparing PI3K signalling in GL cells from women with anovulatory PCOS, who are the most insulin resistant group and had significantly higher testosterone levels, than controls, that there would be a significant difference in Akt phosphorylation between the groups. This was not shown to be the case. No significant differences were seen in Akt or GSK 3β phosphorylation between the groups. However there was a reduction of p42/44ERK phosphorylation in the ovulatory PCOS group compared to controls. The role of MAPK in insulin stimulated glucose metabolism is unclear and the significance of this finding with respect to impaired glucose metabolism in granulosa cells remains to be determined. There is some evidence to link p38 MAPK to insulin stimulated glucose metabolism, but not much evidence to support a role for p42/44 MAPK.

Given that insulin resistance in PCOS appears to be selective, affecting the metabolic but not mitogenic activity of insulin it is surprising that defects in p42/44 MAPK signalling was found in the PCOS patients. This study is the
first to suggest that there may be a difference in MAPK signalling between women with and without PCOS. It would be useful to use specific p42/44 ERK inhibitors to see if this has an effect on glucose metabolism. If no changes were seen using this inhibitor then it may be more likely that an alternative pathway that we have not yet investigated may be involved.

The remaining lysates could be used for immunoblotting for phosphorylated AMPK, phosphorylated PKC and phosphorylated p38 MAPK. We could also use phosphoprotein microarrays to see if any other important proteins in the insulin signalling pathway are phosphorylated. Alternatively, it may be that the western blot techniques used were not sensitive enough to pick up small changes in the PI3K pathway.

This is also the first study to investigate the effect of FSH on glucose metabolism in primary human granulosa cells. As in the mouse ovary, FSH can induce glucose uptake and lactate accumulation but without clear evidence of activation of the PI3-kinase pathway. FSH 20ng/ml did however induce measureable increases in p42/44ERK phosphorylation and significant amounts of progesterone production in all three groups, which may suggest an involvement of the MAPK pathway in glucose metabolism or may simply reflect changes in the pathways involved in FSH-induced progesterone production. Another possibility is that an alternative signalling pathway such as AMPK or atypical PKC is involved in FSH induced glucose metabolism but this has not yet been investigated in our system.

It would be useful to test more doses of FSH in our experiments with primary human granulosa cells, and confirm our findings. It would also be useful to check the mRNA levels of FSH receptor like Gonzalez-Fernandez et al. (256)
or use immunocytochemistry to check for the presence and density of FSH receptors in anovulatory PCOS, ovulatory PCOS and control primary human granulosa cells. If we confirm that the FSH receptor levels are higher in our anovulatory PCOS patients, we could hypothesize that the amount of MAPK phosphorylation in PCOS women is actually reduced considering the quantity of FSH receptors is greater.

It is hoped the greater the knowledge of the specific pathways involved in the dysfunction of granulosa cell glucose metabolism, the greater chance of ultimately developing specific therapeutic agents to improve the ovarian dysfunction seen in PCOS, particularly in anovulatory PCOS, the most insulin resistant group.

This thesis has shown us that selective insulin resistance is present in granulosa cells but the changes in glucose uptake are small and not reflected in obvious changes in activation of the PI3K pathway. From a clinical and scientific point of view, our findings are significant because there are very dynamic changes in oocyte metabolism, particularly in the maturing follicle and even small changes in insulin-mediated glucose metabolism may have an impact on oocyte function and fertility.

It may also be possible that insulin action is far less important than gonadotrophins in granulosa cell glucose metabolism. We have provided evidence that gonadotrophins are involved in glucose metabolism in granulosa cells, and may well be the primary regulator of glucose metabolism in these cells. The pathways stimulated by FSH stimulated have also been explored and did not reflect any obvious changes in activation of the PI3K pathway.
Conclusion

So where does this research take us? Further study of FSH and LH regulation of glucose metabolism in the human ovary should be investigated in detail in the normal ovary. Comparisons can then be made between gonadotrophin action on glucose metabolism in the normal and PCOS ovary.

Insulin resistance in other target tissues such as adipose and skeletal muscle may be even more important areas for research in PCOS. Insulin action on glucose metabolism and signalling pathways in adipose tissue biopsies from women with and without PCOS could be investigated.
Appendices
### Appendix 1: Table of commonly used antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Use</th>
<th>Dilution</th>
<th>Blocker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-phospho-Akt (ser 473) monoclonal</td>
<td>Cell Signalling Technology 4060 New England Biolabs, Hertfordshire, UK</td>
<td>WB</td>
<td>1:1000</td>
<td>5% non fat milk/TBS 0.15% TWEEN 20</td>
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<tr>
<td>Akt</td>
<td>Cell Signalling Technology 9272 New England Biolabs, Hertfordshire, UK</td>
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<td>1:1000</td>
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<tr>
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</tr>
<tr>
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Western blotting (WB), Immunoprecipitation (IP), Immunofluorescence (IF), Immunocytochemistry (ICC)
## Appendix 2: Table of commonly used solutions

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</thead>
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<td>20% Glycerol</td>
<td>121g Tris base</td>
</tr>
<tr>
<td>2% SDS</td>
<td>578g Glycine</td>
</tr>
<tr>
<td>0.1M Tris pH 6.8</td>
<td>40g SDS</td>
</tr>
<tr>
<td>10% β-Mercaptoethanol</td>
<td>Water to 4 litres</td>
</tr>
<tr>
<td>7M Urea</td>
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</tr>
<tr>
<td>0.004% β-mercaptoethanol</td>
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</tr>
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<td>Urea</td>
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<th>Laemmli Loading Buffer</th>
<th>RIPA/SDS</th>
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<tr>
<td>20% Glycerol</td>
<td>1% Nonidet P-40</td>
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<tr>
<td>2% SDS</td>
<td>1% Triton X-100</td>
</tr>
<tr>
<td>0.1M Tris pH 6.8</td>
<td>1% Sodium deoxycholate</td>
</tr>
<tr>
<td>10% β-Mercaptoethanol</td>
<td>0.1% SDS</td>
</tr>
<tr>
<td>7M Urea</td>
<td>150mM NaCl</td>
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<tr>
<td>0.004% bromophenol blue</td>
<td>10mM Tris pH 8.0</td>
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<tr>
<td>0.004% b-mercaptoethanol</td>
<td>2nM NaF</td>
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<td>RIPA/SDS was stored at 4°C and 10μl per ml of protease inhibitor mix and Aprotinin was added before use</td>
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<th>Transfer Buffer</th>
<th>Protease inhibitor mix</th>
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<td>25mM Tris base</td>
<td>20mg/ml phenyl methyl sulfonyle fluoride (PMSF)</td>
</tr>
<tr>
<td>0.2M Glycine</td>
<td>20mg/ml 1-10 phenanthroline</td>
</tr>
<tr>
<td>20% Methanol</td>
<td>20mg/ml Benxamine</td>
</tr>
<tr>
<td>0.05% SDS</td>
<td>Dissolved in ethanol and stored at -20°C</td>
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<table>
<thead>
<tr>
<th>Polyacrylamide 12% gel</th>
<th>Polyacrylamide stacking gel</th>
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<tbody>
<tr>
<td>10.15 ml Deionised water</td>
<td>6.8 ml Deionised water</td>
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<tr>
<td>20 ml 30% Acrylamide / Bis solution</td>
<td>1.7 m 30% Acrylamide / Bis solution (Biorad)</td>
</tr>
<tr>
<td>18.75 ml 1M Tris (pH 8.8)</td>
<td>1.25 ml 1 M Tris (pH 6.5)</td>
</tr>
<tr>
<td>0.5 ml 10 % SDS</td>
<td>0.1 ml 10 % SDS</td>
</tr>
<tr>
<td>0.5 ml 10 % APS</td>
<td>0.1 ml 10 % APS</td>
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<tr>
<td>30 μl TEMED</td>
<td>10 μl TEMED</td>
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<table>
<thead>
<tr>
<th>Polyacrylamide 8% gel</th>
<th>2xTY + AGAR</th>
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<tbody>
<tr>
<td>16.95 ml Deionised water</td>
<td>Same as 2xTY but with 15g of Bacto-agar add per litre.</td>
</tr>
<tr>
<td>13.4 ml 30% Acrylamide / Bis solution</td>
<td></td>
</tr>
<tr>
<td>(Biorad)</td>
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</tr>
<tr>
<td>18.75 ml 1M Tris (pH 8.8)</td>
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</tr>
<tr>
<td>0.5 ml 10 % SDS</td>
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<tr>
<td>0.5 ml 10 % APS</td>
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<td>30 μl TEMED</td>
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| 2xTY | |
|-------||
| 16g Tryptone | |
| 10g Bacto-yeast extract | |
| 5g NaCl | |
| Water to 1L | |
Appendix 3: Stimulants and inhibitors used for cell treatment in protein expression assays

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<tr>
<th>Stimulants and inhibitors</th>
<th>Supplier</th>
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<tr>
<td>Insulin</td>
<td>Sigma, Dorset, UK</td>
<td>10mg/ml in acidified H2O</td>
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<tr>
<td>Dihydrotestosterone</td>
<td>Sigma, Dorset, UK</td>
<td>5mg/ml in 100% ethanol</td>
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<tr>
<td>FSH (recombinant peptide hormone)</td>
<td>National hormone and pituitary program, California, USA</td>
<td>1IU/µL, 100ng/µL in PBS 0.1% BSA mixture</td>
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<tr>
<td>Wortmannin</td>
<td>Sigma, Dorset, UK</td>
<td>0.1mg/ml in 10% ethanol</td>
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<tr>
<td>Flutamide</td>
<td>Sigma, Dorset, UK</td>
<td>50mg/ml in 100% ethanol</td>
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<tr>
<td>LY 294002</td>
<td>Sigma, Dorset, UK</td>
<td>5mg/ml in DMSO</td>
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## Appendix 4: Programmes used for running the COBAS BIO Autoanalyser

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Appendix 5: Summary of total patients recruited.

Complete data: Western/glucose uptake/lactate depletion and progesterone production:

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Incomplete data sets:

- Progesterone missing in Clinical Biochemistry (4)
- No FSH data, No 1ng/ml Insulin data (4)
- Insufficient cells, unable to analyse (8)
- No glucose or lactate data insufficient cells (2)
- Infection cells thrown away (4)
- Patients used in preliminary studies plating cells/technique/doses/manual mistakes (11)
- Controls used for insulin vs insulin and androgen expt (2)
Abbreviations
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References


44. Wachs DS, Coffler MS, Malcom PJ, Shimasaki S, Chang RJ. Increased androgen response to follicle-stimulating hormone administration in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2008 May;93(5):1827-33.


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