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**Luteinizing hormone/chorionic gonadotropin receptor overexpressed in
granulosa cells from polycystic ovary syndrome ovaries is functionally
active**

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Short Title: Active LHCGR over-expression in PCOS

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Abstract

Polycystic ovarian syndrome (PCOS) is associated with anovulatory infertility. Luteinizing hormone/chorionic gonadotropin receptor (LHCGR), which is critical for ovulation, has been suggested to be expressed prematurely in the ovarian follicles of women with PCOS. The objective of this study was to analyse the expression and activity of LHCGR in ovarian granulosa cells from PCOS patients and the involvement of ARF6 small GTPase in LHCGR internalisation. Granulosa cells (GCs) isolated from follicular fluid collected during oocyte retrieval from normal women (n=19) and women with PCOS (n=17) were used to study differences in LHCGR protein expression and activity between normal and PCOS patients. LHCGR expression is up-regulated in GCs from PCOS women. LHCGR in PCOS GCs is functionally active as evidenced by increased cAMP production upon human gonadotrophin (HCG)-stimulation. Moreover, ARF6 is highly expressed in GCs from PCOS patients and HCG-stimulation increases the levels of active ARF6. The inhibition of ARF6 activation attenuates HCG-induced LHCGR internalisation in both normal and PCOS GCs, indicating that there are no alterations in LHCGR internalisation in GCs from PCOS. In conclusion, the expression and activation of LHCGR and ARF6 are up-regulated in GCs from PCOS women but the mechanism of agonist-induced LHCGR internalisation is unaltered.

KEYWORDS: LHCGR; cAMP; ARF6; PCOS; human granulosa cells.

Introduction

PCOS is one of the commonest disorders of ovulation affecting 5–10% women of reproductive age (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). It is characterised by ovulation failure, polycystic ovaries and excessive androgen production, which lead to infertility (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). PCOS is a complex syndrome having endocrine and metabolic abnormalities. One of the characteristic endocrine defects is the inappropriate exposure of granulosa cells (GCs) of immature follicles to raised levels of luteinizing hormone (LH). The elevated LH may prevent follicular maturation and contribute to failure to ovulate (Yong et al., 1992). LH and chorionic gonadotrophin (CG) share the same receptor (LHCGR) to activate the $G\alpha_s$ subunit of the G-protein/adenylyl cyclase (AC)/cAMP pathway (Choi and Smitz, 2014). Additional coupling of LHCGR through Gq proteins activates phospholipase C, protein kinase B and ERK1/2 pathways (Choi and Smitz, 2014). LHCGR is overexpressed in PCOS GCs, which tend to be hyper-sensitive to LH stimuli with excessive androgen production (Ehrmann et al., 1995). Moreover, previous studies have suggested that LHCGR, which is critical for ovulation, may be induced prematurely in women with PCOS (Jakimiuk et al., 2001, Willis et al., 1998). Despite a plethora of studies on clinical, metabolic, genetic and evolutionary factors in PCOS (Gorry et al., 2006, Ben-Shlomo and Younis, 2014, Corbett and Morin-Papunen, 2013, Williams et al., 2013), only few studies have advanced our understanding of the physiopathology of this disorder. A specific clinical risk in PCOS is the development of ovarian hyperstimulation syndrome (OHSS) due to excessive and unpredictable response to gonadotrophin stimulation (Swanton et al., 2010), hence studies on the regulation of LHCGR response are needed to increase our understanding of PCOS and to identify biological mechanisms that might explain the severity of OHSS in women at risk.

After agonist stimulation, LHCGR internalises through ARF6 small GTPase mediated β -arrestin-clathrin-dynamin pathway (Kanamarlapudi et al., 2012b, Hunzicker-Dunn et al., 2002). LHCGR internalisation and recycling regulate the density of cell surface receptors and thereby the sensitivity of the cells to LH (Bhaskaran and Ascoli, 2005). ARF6 is a member of the ARF family of small GTPases, which regulates membrane trafficking by cycling between the active GTP- and inactive GDP-bound states (Donaldson and Jackson, 2011). ARFs are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs). Among the six known mammalian ARF isoforms (ARFs1-6), ARF1 and ARF6 are the best characterised. ARF1 localises to and acts at the Golgi whereas ARF6 localises to and acts at the cell periphery. ARF6 mediates cell surface receptor internalisation and the actin cytoskeleton reorganisation beneath the plasma membrane (Donaldson and Jackson, 2011). The activation of ARFs1-5, but not ARF6, is inhibited by the fungal toxin brefeldinA (BFA). However, ARF6 activation by the cytohesin family of ARF GEFs is inhibited by a chemical inhibitor secinH3 (Hafner et al., 2006).

In this manuscript, we have focused on functional mechanisms of gonadotrophin response in PCOS GCs to complement previous studies on clinical and genetic factors. Our findings in this manuscript are relevant both to the physiology of GC function and to the pathophysiology PCOS. The aim this study was to analyse the expression and activity of LHCGR and ARF6, and measured ARF6-mediated LHCGR internalisation in GCs from normal and PCOS women. This would help in understanding the relationship between OHSS and PCOS. This analysis revealed that LHCGR and ARF6 expression and activity are increased in PCOS GCs but there is no change in agonist-induced LHCGR internalisation.

Materials and Methods

GCs isolation and culture

GCs were obtained from follicular fluid collected during oocyte retrieval from women undergoing in vitro fertilisation (IVF)/intracytoplasmic sperm injection (ICSI) at the Bristol centre for Reproductive Medicine. Follicular fluid aspirated is an excellent source of granulosa-lutein cells that retain functional Gs-adenylyl cyclase and Gq-phospholipase C coupling (Asboth et al., 2001, Carrasco et al., 1997). To obtain a sufficient number of GCs for some experiments, follicular fluid from several individual follicles were combined. The normal group included 19 women (age range 25-43, mean 34.2 years) undergoing IVF/ICSI with regular menses, unexplained infertility, tubal factor or male factor infertility and normal ovarian morphology on ultrasound. The PCOS group comprised 17 women (age range 24-41, mean 35.6 years) with irregular periods, infertility, hyperandrogenism and morphological appearance of polycystic ovaries; the diagnosis was made according to the established guidelines (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). The body mass index (BMI; kg/m²) in the normal (22.5±7) and PCOS (23.3±3.5) groups was similar. The duration of infertility at 48 months (range 36-72) was significantly longer in the PCOS group than in the normal group (36, range 24-48 months; p = 0.006). Serum FSH (IU/L) levels were similar in the two groups: 6.5±1.6 versus 6±1.3 for normal and PCOS, respectively. The study was conducted with local research ethics committee approval and written informed consents were obtained from all participants.

Both groups of women received gonadotrophin releasing hormone analogue nasal spray (Suprefact, Aventis Pharma, Kent, UK) for pituitary desensitization. Serum oestradiol was measured to confirm pituitary suppression. Following this, follicle-stimulating hormone (human menopausal gonadotrophin Menopur, Ferring, UK) was used to induce follicular maturation, which was monitored with vaginal ultrasound scans. The total dose of FSH (IU) used was 2428 ± 978 for the normal group and 2536 ± 1189 for the PCOS group; the

difference was not statistically significant. When at least three follicles reached the size of ≥ 17 mm, 6500IU recombinant HCG (Ovitrelle, Merck Serono, Feltham, UK) was administered to complete follicular/oocyte maturation. Transvaginal ultrasound oocyte retrieval was undertaken 36h after HCG administration. The clinical pregnancy rates were similar in both normal and PCOS groups (39% in women under 35 years of age and 24% in women over 39 years old).

GCs isolated from the pooled follicular fluid and washed twice with PBS containing 0.5% bovine serum albumin (PBS-BSA) by centrifugation at 400g for 10 min. The GCs resuspended in 10ml Dulbecco's modified Eagle's medium (DMEM) and overlaid onto 12.5ml sterile 35% Percoll in PBS-BSA and centrifuged for 30min at 400g to obtain GCs as the layer at the interface of medium and percoll (Asboth et al., 2001). GCs were then washed three times with PBS-BSA and re-suspended in DMEM containing 2mM glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin and 10% fetal calf serum (complete medium) and determined cell number and viability by trypan blue (0.4%) staining. We routinely obtain $1.5-3 \times 10^6$ cells with 75-85% viability per sample by this method. GCs were plated in 24-well (8×10^4 cells per well) or 30mm plates (4×10^5 cells) in complete medium and cultured 4 days in a humidified incubator at 37°C/5% CO₂ (Yong et al., 1992, Schipper et al., 1993).

Cyclic AMP assay

GCs cultured in 24-well plates were used for cAMP estimation as described (Kanamarlapudi et al., 2012b). Briefly, GCs were serum starved for 2h and incubated with 0.25mM of phosphodiesterase inhibitor Ro201724 at 37°C for 15min. The cells were then stimulated with HCG 10-1000IU/ml for 30min or 10IU/ml for 0-60min at 37°C. Where indicated, cells were incubated with the inhibitors for indicated time at 37°C prior to stimulation with HCG. The reaction was terminated by adding 4% (w/v) ice-cold trichloroacetic acid and the cAMP

content of the samples was analysed by a cAMP binding protein assay as described previously (Lawrence et al., 2005).

Preparation of the cell lysates

GCs cultured in 30mm plates were lysed using 0.2ml of ice-cold modified RIPA lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% Triton x-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10mM MgCl₂) with 1% mammalian protease inhibitor mix (Sigma-Aldrich, Dorset, UK). The protein content of cell lysates was estimated using the bicinchoninic acid (BCA) assay (Sigma-Aldrich, Dorset, UK) and BSA as a protein standard (Thompson and Kanamarlapudi, 2014).

Active ARF6 pull down assay

ARF6 activation was assessed by using the GST-GGA3 protein binding domain (PBD; amino acids 1–316) pulldown assay (Kanamarlapudi, 2014). The GST-GGA3 PBD fusion protein purified and coupled to glutathione beads as previously described (Venkateswarlu et al., 1998). GCs isolated from PCOS and normal women were serum starved for 2h and stimulated without or with HCG 10IU/ml for 30min at 37°C. The cells were then lysed using ice-cold modified RIPA lysis buffer with 1% mammalian protease inhibitors mix. The cell lysates were incubated with glutathione-Sepharose beads coupled to 50µg of purified GST-GGA3 PBD fusion protein at 4°C for 2h. The beads were washed three times with the wash buffer (50mM Tris-HCl, pH 7.5, 10mM MgCl₂, 150mM NaCl and 1% Triton X-100). The lysates that not incubated with the beads were used as input controls. The active ARF6 bound to the beads and total ARF6 in the inputs were determined by immunoblotting using an anti-ARF6 antibody. Immunoblots were scanned and the active GTP-bound ARF6

precipitated with GST-GGA3 PBD beads was normalised to total ARF6 levels in the lysates to compare ARF6-GTP levels in GCs from normal and PCOS women.

Immunoblotting

This was carried out as described previously (Kanamarlapudi et al., 2012a). Briefly, proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto polyvinylidene fluoride membrane. Membranes were blocked with TBS-T (TBS with 0.1% tween-20) containing 5% skimmed milk (blocking buffer) for 1h at room temperature. Membranes were immunoblotted with primary antibody diluted in blocking buffer (an anti-LHCGR [1:1000 dilution] or an anti-ARF6 mouse monoclonal [1:200 dilution] or an anti-ARF1 rabbit polyclonal [1:2500 dilution] or an anti- α tubulin mouse monoclonal [1: 10000 dilution]) for 1h at room temperature. Membranes were washed three times with TBS-T and then incubated with the HRP-conjugated anti-mouse or anti-rabbit secondary antibody (diluted 1:2500 in blocking buffer) for 1h at room temperature. Membranes were washed three times with TBS-T and then incubated in ECL select substrate (GE Healthcare, Hertfordshire, UK), and bands visualised using the ChemiDoc™ XRS system (Bio-Rad, Herts, UK) (Thompson and Kanamarlapudi, 2015a).

Data Analysis

Image-J program was used for densitometric analysis. Data were analysed by using the GraphPAD Prism program. Results are expressed as the mean \pm standard error of the mean (SEM) of three to five experiments. Statistical significance between multiple groups was determined by the Bonferroni's post hoc test after one-way or two-way analysis of variance (ANOVA), where $p < 0.05$ was considered as statistically significant (Thompson and Kanamarlapudi, 2015b).

Results

LHCGR protein expression in GCs from normal and PCOS women was analysed by immunoblotting. The LHCGR protein was detected as an 85kDa band in both normal and PCOS GCs (**Figure 1A**). The PCOS group showed a more intense band than the normal group, indicating over-expression of LHCGR protein in PCOS GCs. LHCGR protein expression in GCs from PCOS women is ~150% higher than in normal GCs (**Figure 1B**). We next determined whether LHCGR over-expressed in PCOS GCs is functionally active. Since agonist-stimulated LHCGR signals through cAMP to cause steroidogenesis in GCs, we measured cAMP production in response to HCG-stimulation. The HCG-induced cAMP accumulation was measured in GCs from PCOS and compared to that in the normal group. There was a dose- and time-dependent increase in cAMP production in both groups, and the cAMP accumulation in GCs from PCOS group was significantly higher than in GCs from normal individuals (**Figure 2**). Moreover, basal cAMP production in GCs from PCOS was higher than that in normal responders. The increase in both basal and HCG-stimulated cAMP levels observed in GCs from PCOS compared to that in normal GCs indicated that LHCGR over-expressed in PCOS ovaries is functionally active. .

ARF6 small GTPase plays a crucial role in agonist-induced LHCGR internalisation whereas ARF1 is important for protein trafficking at the Golgi (Hunzicker-Dunn et al., 2002, Kanamarlapudi et al., 2012b, Donaldson and Jackson, 2011). To determine whether the expression of ARF1 and/or ARF6 is altered in PCOS, the expression of ARF1 and ARF6 proteins in GCs from normal and PCOS women was analysed by immunoblotting. ARF1 and ARF6 expression was detected in both normal and PCOS GCs. The PCOS group showed a more intense band of ARF6 than the normal group, indicating overexpression of ARF6 in PCOS GCs (**Figure 3A**). However, ARF1 expression in GCs from PCOS is similar to that in normal GCs, indicating no change in ARF1 expression in PCOS GCs. ARF6 activation by

LHCGR stimulation in GCs of PCOS and normal groups was investigated by precipitating ARF6-GTP from the lysates of GCs of both groups using GST-GGA3 PBD coupled glutathione beads. The basal ARF6-GTP levels in GCs of PCOS group were higher than that in the normal group GCs (**Figure 3B**). The agonist-stimulated GCs showed a significant but similar increase in ARF6-GTP levels (~3 fold over basal) in both the groups.

Since ARF6 plays an important role in LHCGR internalisation, we determined the effect of ARF6 inhibitory peptide (Myr-ARF6p) on cAMP accumulation, which is readout for the LHCGR internalisation, in GCs from both the groups. GCs from PCOS and normal responders pre-incubated with inhibitory membrane permeable (through penetratin-coupling) Myr-ARF6p or Myr-ARF1p or control penetratin peptide were stimulated with agonist and cAMP accumulation was measured. Myr-ARF6p, but not Myr-ARF1p, treatment increased cAMP accumulation over basal to a similar level in both groups, suggesting that there is no alteration in LHCGR internalisation in GCs from PCOS (**Figure 4A**). ARF6 function depends on its activation by GEFs such as cytohesins (Davies et al., 2014a, Davies et al., 2014b). We and others have shown the involvement of cytohesin2, through ARF6 activation, in agonist-induced internalisation of LHCGR (Hunzicker-Dunn et al., 2002, Kanamarlapudi et al., 2012b). We investigated here whether the chemical inhibitor of cytohesin family of ARF GEFs, secinH3 (Hafner et al., 2006), inhibits LHCGR internalisation and thereby increases cAMP production in GCs (**Figure 4B**). SecinH3, but not BFA (which inhibits the activation of ARFs1-5 but not ARF6), caused significant increase in HCG-induced cAMP levels in both groups, suggesting that there are no alterations in LHCGR internalisation in PCOS GCs.

Discussion

In women, PCOS is the major cause of infertility and other consequences ranging from hirsutism and acne first appearing during adolescence to long-term health risks, in particular a significant increase in the risk of developing type II diabetes in later life (Gorry et al., 2006, Wang et al., 2010). Although the clinical and biochemical features of PCOS are well described, our paper provides novel and unique insight into the regulation of LHCGR receptor in granulosa cells of normal and PCOS women. Women with PCOS are at greater risk of developing OHSS after controlled ovarian hyperstimulation using gonadotropins for IVF (Schmidt et al., 2014). We provide evidence here that overexpression of functional LHCGR in GCs of PCOS is likely to be part of the mechanism of OHSS. Moreover, previous studies suggested that LHCGR may be expressed prematurely in women with PCOS (Jakimiuk et al., 2001, Willis et al., 1998). Interestingly, a number of genes involved in gonadotrophic response, steroidogenesis and water permeability e.g. growth factors (*BMP6*), amphiregulin (*AREG*) and aquaporins (*AQP3*) are upregulated in PCOS follicles (Schmidt et al., 2014) but the functional significance of these changes remains to be investigated.

We showed in this study that LHCGR protein is overexpressed in GCs from PCOS women. This is consistent with a previous observation (Jakimiuk et al., 2001). The factors regulating LHCGR expression in granulosa cells are not well known. It has been observed that insulin increases FSH and LH action along with increased steroidogenesis (progesterone) in PCOS GCs compared to normal GCs in vitro (Willis et al., 1996). Moreover polymorphisms in the insulin receptor gene have been described in women with anovulatory PCOS (Cui et al., 2015). The increase in insulin levels has also been shown to up-regulate FSH induced LHCGR expression in murine GCs (Eppig et al., 1998). However, although hyperinsulinaemia tends to be present in PCOS women with hirsutism and obesity (Moran and Teede, 2009), the majority of women with PCOS have normal insulin levels (Li et al.,

2014). Further research is necessary into the endocrine and paracrine regulation of LHCGR expression in women, including the role of growth factors and steroid hormones.

Further we have shown here that LHCGR over-expressed in GCs from PCOS patients is functionally active. This is consistent with a previous report that GCs from PCOS patients are more responsive to LH in terms of oestradiol and progesterone production than GCs from normal women (Willis et al., 1998). Increased LHCGR activity may also be responsible for the overexpression of aquaporins in PCOS, given that excessive fluid permeability is one of the features of severe OHSS (Schmidt et al., 2014).

It has been shown previously that ARF6 small GTPase activated by LHCGR through cytohesin family ARF GEFs mediates the receptor internalisation and signalling (Kanamarlapudi et al., 2012b). Since LHCGR is overexpressed in PCOS group, we also analysed, in this study, whether LHCGR overexpression has any effect on ARF6 small GTPase expression and activity in PCOS GCs. Our analysis revealed that ARF6 expression and its basal activity are higher in PCOS group. However, HCG-stimulated ARF6 activation is similar in both normal and PCOS groups. Consistent with this result, the ARF6 specific GEF, cytohesin2, overexpression in mature pre-ovulatory follicles has been reported (Mukherjee et al., 2001). Therefore it is possible that cytohesin 2 mediates ARF6 activation downstream of LHCGR. LHCGR internalises following its activation, which regulates the biological responsiveness of the receptor (Bhaskaran and Ascoli, 2005). The inhibition of LHCGR internalisation increases the density of cell surface receptors and thereby AC activation with consequent increase in cAMP accumulation. Therefore alterations in LHCGR internalisation could result in ovarian hyper-stimulation. However, we have shown in this study that LHCGR internalisation is unaltered in the PCOS group.

Our study has limitations in that we used granulosa cells following gonadotropin stimulation for IVF. We did not have access to granulosa cells from very small follicles and

the important paracrine interactions between granulosa and theca cells could not be studied. It will be important in future research to establish the role of ARF6 in LHCGR function in the early stages of follicular selection and development, which can be altered in women with PCOS. Another important area for future research is the interaction between ARF6/cytohesins and the vascular endothelial growth factor (VEGF), given the perceived involvement of VEGF in the vascular permeability alterations in women with OHSS (Mannell et al., 2012, Peitsidis and Agrawal, 2010).

In summary, we provide for the first time evidence that LHCGR overexpressed in women with PCOS is functionally active and that ARF6 small GTPase expression is increased in ovaries from women with PCOS. Furthermore, HCG-stimulation increased ARF6 activation in GCs from PCOS ovaries. The inhibition of ARF6 activation reduced HCG-induced LHCGR internalisation in both normal GCs and PCOS GCs, suggesting that there are no changes in LHCGR internalisation in women with PCOS. This study highlights the cellular pathways involved in causing the hyper-response to gonadotropins in women with PCOS.

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FIGURE LEGENDS

Figure 1. **The expression of LHCGR in GCs from normal and PCOS ovaries.** A. Immunoblot analysis of LHCGR expression in GCs isolated from normal and PCOS ovaries. GCs were cultured in vitro for 4 days, lysed and the lysates were immunoblotted using a rabbit polyclonal LHCGR antibody (α -tubulin was used as loading control). B. Corresponding quantitative data for A. The intensity of the bands in A was quantified and normalised to the expression of loading control (α -tubulin). Values are mean \pm SEM from five individual experiments (***, $P < 0.001$).

Figure 2. **The activity of LHCGR in normal and PCOS GCs.** Dose- (A) and time-dependent (B) accumulation of cAMP in normal (Δ) and PCOS (\blacktriangle) GCs. GCs cultured in a 24-well plate were serum starved for 2h and stimulated with 1-1000 IU/ml of HCG for 30min or 10 IU/ml for 0-60min and cAMP levels were measured in the cultured media. Values are mean \pm SEM from five individual experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3. **The expression and activity of ARF6 in GCs from normal and PCOS.** A. Immunoblot analysis of ARF1 and ARF6 protein expression in GCs isolated from normal and PCOS women. The intensity of the bands was quantified, normalised to the expression of loading control (α -tubulin) and shown below the respective blots. B. The basal and HCG-induced ARF6 activation (ARF6-GTP) in PCOS and normal GCs. GCs isolated from PCOS and normal women were serum starved for 2h and stimulated without or with HCG 10 IU/ml for 30min at 37°C. The cell lysates were incubated with GST-GGA3 PBD resin and the protein bound to the resin was analysed by immunoblotting using an anti-ARF6 antibody. Values are mean \pm SEM from three individual experiments (*, $P < 0.05$; ***, $P < 0.001$).

Figure 4. **The effect of ARF6 activation inhibitors on cAMP accumulation in normal and PCOS GCs.** The Myr-ARF6 inhibitory peptide (**A**) and SecinH3 (**B**) effect on cAMP accumulation in HCG-stimulated GCs from PCOS women was assessed. The GCs from normal and PCOS women cultured in a 24-well plate were incubated in triplicate with indicated inhibitor in DMEM for 2h at 37°C and stimulated with HCG 10 IU/ml for 30min. The cAMP levels were then measured in the culture media. Values are mean \pm SEM from three individual experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).