Gene expression differences induced by equimolar low doses of LH or hCG in combination with FSH in cultured mouse antral follicles

Ingrid Segers, Tom Adriaenssens, Sandra Wathlet and Johan Smitz
Follicle Biology Laboratory, Vrije Universiteit Brussel, Laarbeeklaan 101, 1090 Jette, Belgium
(Correspondence should be addressed to I Segers; Email: segersingrid@gmail.com)

Abstract

In a natural cycle, follicle growth is coordinated by FSH and LH. Follicle growth stimulation in Assisted Reproductive Technologies (ART) requires antral follicles to be exposed to both FSH and LH bioactivity, especially after GnRH analog pretreatment. The main aim was to detect possible differences in gene expression in granulosa cells after exposing the follicle during antral growth to LH or hCG, as LH and hCG are different molecules acting on the same receptor. Effects of five gonadotropin treatments were investigated for 16 genes using a mouse follicle culture model. Early (day 6) antral follicles were exposed to high recombinant FSH combined or not with equimolar concentrations of recombinant LH (rLH) or recombinant hCG (rhCG) and to highly purified human menopausal gonadotropin (HP-hMG) for 6 h, 12 h, or 3 days. Expression differences were tested for genes involved in steroidogenesis: Mok, Lss, Cyp11a1, Hsd3b1, Cyp19a1, Nr4a1, and Timp1; final granulosa differentiation: Lhgr, Oxtr, Pgr, Egfr, Hif1a, and Vegfa; and cytokines: Cxcl12, Cxcr4, and Sdc4. Lhgr was present and upregulated by gonadotropins. Nr4a1, Cxcl12, and Cxcr4 showed a different expression pattern if LH bioactivity was added to high FSH in the first hours after exposure. However, no signs of premature luteinization were present even after a 3-day treatment as shown by Cyp19a1, Oxtr, Pgr, and Egfr and by estrogen and progesterone measurements. The downstream signaling by rhCG or rLH through the LHCR was not different for this gene selection. Granulosa cells from follicles exposed to HP-hMG showed an enhanced expression level for several genes compared with recombinant gonadotropin exposure, possibly pointing to enhanced cellular activity.


Introduction

The final stages of follicle development leading to ovulation are dependent on two pituitary glycoproteins: FSH and LH. Granulosa cells of primary follicles start expressing FSH receptors (Fshr) and the theca cells of secondary follicles start expressing LH receptors (Lhgr) (Erickson et al. 1985, Oktay et al. 1997). Early antral follicle growth is only sustained under rising FSH serum concentrations, which induce a rise in estradiol (E2) and inhibin B (Inhb) (Groome et al. 1996), followed by expression of Lhgr on granulosa cells (Peng et al. 1991). Negative feedback mechanisms of E2 and INHB on FSH concentrations select out a dominant follicle, which can survive due to its LH responsiveness (McGee & Hsueh 2000). Stimulation of ovarian follicle growth in human ART practice involves the use of gonadotropin preparations containing either FSH alone or combinations of FSH plus LH activity. LH activity in highly purified human menopausal gonadotropin (HP-hMG) is mainly represented by hCG stimulation (Wolfenson et al. 2005). More recently, recombinant LH (rLH) became available to complement recombinant FSH (rFSH) during stimulation (Mochtar et al. 2007). Differences in gene expression in human follicle cells were shown in relation to the type of gonadotropin preparation used (Grondahl et al. 2009, Adriaenssens et al. 2010), and these might influence clinical outcome (Afnan 2009, van Wely et al. 2011). Also the origin of the gonadotropin preparation, urinary or recombinant, might, via isoform composition differences, affect bioavailability and bioactivity in relation to the species (de Leeuw et al. 1996).

Gonadotropin receptors typically activate adenyl cyclase through G-proteins and thereby induce cAMP production. However, the cellular response upon FSH cannot entirely substitute for LHCR signaling during the final stages of follicle growth and ovulation as shown in the Lhgr knockout mouse (Zhang et al. 2001) and in women with inactivating mutations of Lhgr (Huhtaniemi & Alevizaki 2006). Hence, stimulation of FSHR or LHCR during antral follicle growth leads to a specific differential response.

The large N-terminal ectodomain of the LHCR is responsible for the high affinity and selective binding of its two ligands LH and hCG (Caltabiano et al. 2008). The LH and hCG β subunits share 80% sequence homology but greatly differ in carbohydrate additions (for review Fares (2006)). Where the conserved amino acid composition of LH and hCG determines the ligand–receptor specificity, the divergent
carbohydrate chains of LH and hCG interact differently with the LHCGR changing their affinity to the receptor and affect half-life and hence bioactivity in living organisms (Galet & Ascoli 2005). Although in vivo bioactivity will largely determine differences in the extent of LHCGR stimulation upon LH or hCG stimulation, it remains to be determined whether LH and hCG can elicit intrinsically different responses at the level of the Lhgr.

Our primary interest was to differentiate low-dose effects of LH and hCG on in vitro cultured follicles exposed to a high-FSH dose (25 mIU/ml) comparable with a superovulation condition. The validated mouse model used is physiologically relevant as it allows natural interactions between the three cell types of the follicle (Cortvrindt & Smutz 2002). The responsiveness and sensitivity for FSH and LH in this in vitro follicle model has already been studied (Cortvrindt et al. 1998, Adriaens et al. 2004). Different gonadotropin regimens were shown to induce differences in mRNA expression in follicle cells (Adriaenssens et al. 2009, Sanchez et al. 2011) and in secretion of proteins (Foster et al. 2010). Therefore, in the current study, early antral follicles were exposed to five different gonadotropin regimens relevant to the ART clinic. A low rFSH tonus (10 mIU/ml) was used during the preantral follicle stage (from days 1 to 6), followed by a high FSH supplementation (as in clinical ART, 25 mIU/ml) in combination or not with low doses of LH or hCG or by HP-hMG. The doses of LH or recombinant hCG (rhCG) included (5–35 pM, equivalent to 5 mIU/ml rhCG) were equimolar and similar to the amount of LH/hCG present in HP-hMG, when the dose used in medium is 25 mIU/ml. These doses are 240 times less than the ovulatory trigger (1·2 IU/ml hCG) and ~100 times less than the minimal effective dose (0·4 IU/ml rhCG, historical laboratory data) to induce mucification and maturation in the applied follicle culture system.

Downstream acute effects of gonadotropin exposure were studied by quantitative gene expression (day 6 at 6 and 12 h) and the chronic effects after 3 days of exposure. The 16 genes chosen for analysis involve LH-induced processes, e.g. steroidogenesis: Mok, Lss, Cyp11a1, Hsd3b1, Cyp19a1, Nr4a1, and Timp1; final differentiation of granulosa cells: Lhgr, Oxtr, Pgr, Egfr, Hif1a, and Vegfa; and cytokine expression: Cxcl12, Cxcr4, and Sdc4 (Table 1).

Materials and Methods

Mice and follicle culture

F1 mice (C57BL/6J×CBA/Ca; Charles River, Brussels, Belgium), housed and bred according to the national standards for animal care and approved by the Ethical Committee for animal experiments of the Free University Brussels (Project 09–216–1), were used in this study. Preantral follicles (110–130 μm) were mechanically isolated from ovaries from 13- to 14-day-old F1 mice in L15 Leibovitz–glutamax–I medium supplemented with 10% heat-inactivated fetal bovine serum (HIA FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (all Gibco; Invitrogen) and placed as single units in half area 96-well plates (Costar; Elscolab, Kruibeke, Belgium). On the first day of culture, intact oocyte–granulosa cell connections and presence of theca cells were ascertained. At days 6, preantral follicles that developed into early antral follicles were considered for exposure to the five treatments during their antral growth phase. At day 9, these follicles had developed into late antral follicles that produced mature oocytes and expanded cumulus cells 16 h after maturation induction. Culture medium was refreshed every 3 days by sampling 30 μl from 75 μl culture medium and adding 30 μl fresh medium. Preantral follicles were cultured for 6 days in a basal culture medium supplemented with 10 mIU/ml rFsh (‘Gonal F’, Ares Serono). Basal culture medium consists of 2% minimal essential medium with glutamax–I (Gibco; Invitrogen) supplemented with 5% HIA FBS, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium (Sigma). Gonadotropin products were dissolved in basal medium. At day 9 of culture, follicles in reference plates received 1·2 IU/ml rhCG (‘Ovidrel’, Ares Serono) and 4 ng/ml r-EGF (Roche) to induce meiotic resumption. All manipulations were done on a heated stage and cultured follicles were grown at 37 °C, 100% humidity, and 5% CO2 in air.

Experimental design

After the initial growth period of 6 days from the preantral to the early antral follicle stage under a gonadotropin concentration of 10 mIU/ml rFSH, early antral follicles were exposed to five different gonadotropin regimens: 10 mIU/ml rFSH, 25 mIU/ml rFSH, 25 mIU/ml rFSH + 5·35 pM rLH (Luveris, Ares Serono), 25 mIU/ml rFSH + 5·35 pM rhCG, or 25 mIU/ml HP-hMG (‘Menopur’, Ferring). The 10 mIU/ml rFSH condition is a continuation of the minimal effective dose of FSH needed in culture (Adriaens et al. 2004, Segers et al. 2012). The 25 mIU/ml rFSH condition is considered ‘slightly supraphysiological’ in accordance with the supraphysiological FSH injections in assisted reproductive technologies (ART) patients (Sanchez et al. 2011). Supplementation of 25 mIU/ml rFSh with equimolar concentrations of 5·35 pM of rLH (3·66 mIU/ml) or rhCG (5 mIU/ml) was chosen to ensure identical ligand availability (equal to the identical amount of molecules) for the LHCGR in this in vitro system.

The effects of different gonadotropin regimens on gene expression in the mural cell compartment were investigated. Early response on increased doses of gonadotropins (acute phase) was measured at 0, 6, and 12 h after the medium refreshment on day 6. Chronic effects of gonadotropin treatment during early antral to late antral follicle growth were studied by exposure for 3 days up to day 9 of culture. Cells were collected in four sets of experiments. Follicle culture repeats consisted of one of these possibilities: acute phase of
Table 1

<table>
<thead>
<tr>
<th>Gene name (gene symbol)</th>
<th>Function in the ovary</th>
<th>Effect of high doses of LH/hCG (ovulation trigger)</th>
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<tbody>
<tr>
<td>LH/choriogonadotropin receptor (Lhcgr)</td>
<td>Receptor for both LH and hCG, important during antral follicle growth, maturation, and ovulation (McGee &amp; Hsueh 2000)</td>
<td>Lhcgr mRNA is downregulated in rat ovaries (Hoffman et al. 1991)</td>
</tr>
<tr>
<td>Mevalonate kinase (Mvk)</td>
<td>Catalyzes conversion of mevalonic acid to mevalonate-5-phosphate, a step in lanosterol and subsequent cholesterol biosynthesis</td>
<td>Mvk mRNA is transiently upregulated in rat, human, and mouse granulosa cells (Wang &amp; Menon 2005, Wang et al. 2007)</td>
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<tr>
<td>Lanoster synthase (Lss)</td>
<td>Catalyzes conversion of 2,3-oxidosqualene to lanosterol, a step in cholesterol biosynthesis Induced by FSH and inhibited by forkhead box O1 (FOXO1; Liu et al. 2009)</td>
<td>Lanosterol enhanced meiotic resumption in porcine oocytes (Marco-Jimenez et al. 2010)</td>
</tr>
<tr>
<td>Cytochrome P450, family 11, subfamily a, polypeptide 1 (Cyp11a1)</td>
<td>Catalyzes conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of steroid hormones. Increased by FSH and E2 and decreased by FOXO1 (Liu et al. 2009)</td>
<td>Cyp11a1 mRNA is upregulated through EGFR–ERK1/2 signaling in mouse COC (Hernandez-Gonzalez et al. 2006)</td>
</tr>
<tr>
<td>Hydroxy-δ-5-steroid dehydrogenase, 3β- and 3δ-isomerase 1 (Hsd3b1)</td>
<td>Catalyzes conversion of pregnenolone to progesterone Upregulated by FSH and insulin (McGee et al. 1995)</td>
<td>Hsd3b1 mRNA is upregulated in mouse COCs (Hernandez-Gonzalez et al. 2006)</td>
</tr>
<tr>
<td>Cytochrome P450, family 19, subfamily a, polypeptide 1 (Cyp19a1)</td>
<td>Catalyzes conversion of C19 androgens to aromatic C18 estrogens Induced by FSH through the A-kinase pathway (Fitzpatrick &amp; Richards 1991)</td>
<td>Nsr1a mRNA is rapidly and transiently expressed in rat granulosa cells (Park et al. 2001), which inhibits Cyp19a1 expression in a human granulosa-like tumor cell line (Wu et al. 2005)</td>
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<tr>
<td>Nuclear receptor subfamily 4, group A, member 1 (Nr4a1)</td>
<td>Orphan receptor of the steroid hormone receptor superfamily that acts as a nuclear transcription factor implicated in immediate-early response (Stocco et al. 2000)</td>
<td>Pgr mRNA and protein is rapidly and transiently expressed in mouse mural granulosa cells (Robker et al. 2000)</td>
</tr>
<tr>
<td>Progesterone receptor (Pgr)</td>
<td>Essential for follicle rupture during the ovulatory process but does not affect follicle growth, development, differentiation, or luteinization (Robker et al. 2009)</td>
<td>Cxcl12 mRNA and protein is present in human follicle fluid granulosa cells isolated at oocyte pick up (Kryczek et al. 2005)</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 12 (Cxcl12)</td>
<td>Chemokine that mediates chemotaxis and homing of primordial germ cells (Ara et al. 2003), granulosa cell survival in the pre- and peri-ovulatory period (Kryczek et al. 2005), and implantation (Tapia et al. 2008)</td>
<td>Cxcr4 mRNA is transiently induced in mouse COCs (Hernandez-Gonzalez et al. 2006)</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) receptor 4 (Cxcr4)</td>
<td>Receptor for CXCL12 Human CXCR4 expression in cumulus cells is negatively correlated with embryo cleaving capacity (van Montfoort et al. 2008) Induced by hypoxia and EGFR signaling (Phillips et al. 2005) Proteoglycan that directly binds CXCL12 and is involved in Cxcr4-mediated cell invasion (Charnaux et al. 2005)</td>
<td>Sdcd mRNA is progressively upregulated in mouse cumulus cells (Adriaenssens et al. 2010)</td>
</tr>
<tr>
<td>Vascular endothelial growth factor A (Vegfa)</td>
<td>Affects follicle growth (Danforth et al. 2003) and survival (Irusta et al. 2010), ovulation, and corpus luteum formation (Fraser et al. 2005)</td>
<td>Vegfa mRNA shows sustained increase in human luteinized granulosa cells (Koos 1995)</td>
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<td>Tissue inhibitor of metalloproteinase 1 (Timp1)</td>
<td>Secreted protein that inhibits matrix metalloproteinases (MMPs) at ovulation and at corpus luteum formation and regression (Li &amp; Curry 2009)</td>
<td>Timp1 mRNA is rapidly and transiently induced in periovulatory rat granulosa cells (Li &amp; Curry 2009)</td>
</tr>
<tr>
<td>Hypoxia inducible factor 1α subunit (Hif1α)</td>
<td>Forms with HIF-1B subunit: HIF1 transcription factor, stabilized by hypoxia, inducing, e.g. VEGF (Forsythe et al. 1996) and Cxcr4 (Phillips et al. 2005). Under normal oxygen levels, Hif1α can be induced by growth factors (Semenza 2003)</td>
<td>HIF1α is induced in human luteinized granulosa cells (van den Driesche et al. 2008)</td>
</tr>
<tr>
<td>Epidermal growth factor receptor (Egfr)</td>
<td>Receptor for several EGF-like factors (e.g. EGF, EREG, and AREG), important during follicle growth and differentiation, steroidogenesis and maturation/ovulation, where it mediates the LH stimulus (Conti et al. 2006)</td>
<td>Egfr mRNA and protein are downregulated in mouse cumulus (Romero et al. 2011)</td>
</tr>
<tr>
<td>Oxytocin receptor (Oxtr)</td>
<td>G-protein-coupled receptor influencing steroidogenesis, ovulation, luteinization, and luteal regression (Gimpl &amp; Fahrenholz 2001)</td>
<td>Oxtr mRNA is induced in mouse mural granulosa cells but not in cumulus cells (Segers et al. 2012)</td>
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Gene expression analysis

Total RNA was extracted using RNeasy Micro kit (Qiagen) on the QiaCube (Qiagen) following the manufacturer's instructions with addition of an on-column DNase I treatment (27 U/reaction; Qiagen). Total RNA (14 μl) in water was obtained. cDNA synthesis was performed on 10 μl total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) using the blend of oligo(dT) and random hexamers. After 30 min at 48 °C, the obtained 20 μl cDNA was diluted four times with RNase-free water and stored at −80 °C until expression analysis. Negative controls were generated in each run of cDNA synthesis by omitting RT enzyme or RNA.

The PCR primer design was performed using Universal Probe Library (UPL) Software (Roche Diagnostics, Roche Applied Science). Gene expression was detected using SYBR green or UPL fluorescent probes. Primer specificity was ascertained by melting curve analysis if SYBR green detection was performed and by sequencing analysis for both SYBR green and probe detection. Primer and reference sequences are detailed in Supplementary Table 1, see section on supplementary data given at the end of this article. Real-time PCR was performed on LightCycler 480 with 0.6 μM primers (Eurogentec, Liege, Belgium) in LC480 SYBR Master or in LC480 Probes Master (Roche Diagnostics) with 0.01 μM probe (UPL, Roche Diagnostics) and 2 μl cDNA into a total volume of 15 μl. PCR conditions were 10 s at 95 °C followed by 45 cycles of 10 s at 95 °C and 30 s at 60 °C. A log10 dilution series of a synthetic oligonucleotide (Eurogentec) corresponding to the amplicon sequence was simultaneously run to enable quantitative measurement of expression. Samples were run in triplicate for each gene. As endogenous control, Rn18s was detected with SYBR green (Adriaenssens et al. 2009). Relative expression values were calculated for all samples. All control samples appeared negative for real-time PCR assessment of Rn18s and specific genes. The different genes were quantified in the most relevant culture setting: acute phase, chronic phase, or both, based on the literature and in-house micro-array data.

Steroid measurements

Spent medium of follicle culture was pooled per plate at each refreshment day or at the collection time point. Samples were frozen at −20 °C. E2-17β production was measured with the RIA E2−RIA-CT (Biosource, Nivelles, Belgium) having a

Table 2 Gene expression results for the acute phase: early response in early antral follicles (day 6) to different gonadotropin regimes for 6 and 12 h. Values represent relative expression ratios of the specific gene and the endogenous control Rn18s (mean ± S.E.M.)

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>6 h</th>
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<tbody>
<tr>
<td>rFSH10 (6)</td>
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<td>rFSH10 (6)</td>
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<tr>
<td>rFSH25 (6)</td>
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<tr>
<td>rFSH25 + LH (5)</td>
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<tr>
<td>rFSH25 + hCG (6)</td>
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<tr>
<td>HP-hMG25 (6)</td>
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</table>

**Mvk**
- 7.3 ± 0.6
- 12.3 ± 1.0
- 14.8 ± 1.1
- 13.3 ± 2.5
- 13.3 ± 1.5
- 9.9 ± 0.7
- 6.2 ± 0.2
- 6.9 ± 0.3
- 7.7 ± 1.0
- 7.7 ± 0.7
- 7.9 ± 1.1

**Lss**
- 1.4 ± 0.2
- 2.7 ± 0.2
- 3.0 ± 0.2
- 3.1 ± 0.5
- 2.9 ± 0.2
- 2.4 ± 0.2
- 1.4 ± 0.1
- 1.6 ± 0.1
- 1.4 ± 0.2
- 1.5 ± 0.2
- 1.6 ± 0.2

**Timp1**
- 2.2 ± 0.2
- 4.6 ± 0.6
- 5.6 ± 0.7
- 5.5 ± 0.9
- 4.3 ± 0.4
- 3.5 ± 0.2
- 1.6 ± 0.2
- 1.6 ± 0.1
- 1.9 ± 0.2
- 2.3 ± 0.4
- 1.8 ± 0.2

**Vegfa**
- 3.2 ± 0.4
- 4.7 ± 0.6
- 4.2 ± 0.5
- 4.7 ± 0.9
- 4.7 ± 0.5
- 4.1 ± 0.3
- 4.9 ± 0.2
- 6.3 ± 0.3
- 4.3 ± 0.5
- 4.7 ± 0.6
- 5.3 ± 0.8

**Sdit4**
- 9.2 ± 1.2
- 13.8 ± 1.8
- 12.4 ± 1.6
- 12.4 ± 1.6
- 11.1 ± 0.8
- 9.0 ± 0.4
- 8.0 ± 0.1
- 8.8 ± 0.1
- 9.4 ± 1.5
- 10.5 ± 1.3
- 9.0 ± 1.5

A,B Different capital letters in a row define statistical differences between the time points 0, 6, and 12 h in rFSH10 treatment (P<0.05, ANOVA+Tukey). Between brackets, the amount of samples analyzed for gene expression is given.


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sensitivity of 20 pg/l and a total imprecision profile <10% coefficient of variation (CV). Progesterone secretion was determined with Prog-CTRIA (Cis bio International, Gif-sur-Yvette Cedex, France) with a functional sensitivity of 0.5 μg/l and a total imprecision profile <10% CV. E₂ was measured on day 6 of culture before and after acute exposure for 6 and 12 h to the different gonadotropin treatments. The average E₂ production per hour was calculated, taking into account the residual fraction of E₂ present in the well after medium change, e.g. after 6 h: [(E₂ on 6 h)−(E₂ on 0 h×0.6)]/6 h. E₂ and progesterone were measured after chronic exposure for 3 days from days 6 to 9 of culture.

Statistical analysis

Relative expression ratios were log₂ transformed to ensure Gaussian distribution. Per time point, different gonadotropin conditions were subjected to one-way ANOVA + Tukey posttest or unpaired test using GraphPad Prism 4.0 Software (La Jolla, CA, USA). Differences with a P<0.05 are considered statistically significant.

Results

Follicle culture

Early antral follicles on day 6 developed into late antral follicles on day 9 as expected, with extensive granulosa cell proliferation and enlargement of follicular structure. There were no obvious morphological differences between the fully grown follicles generated from any of the gonadotropin regimens at day 9. Antral follicle survival up to day 9 was equally successful in all conditions (>96%; P>0.05) and meiotic maturation observed at 16 h after rhCG plus rEGF was >80% in control plates grown for 9 days in all conditions (P>0.05).

LH/choriogonadotropin receptor expression

All follicles included in our analysis had from day 6 onward a clear delineated COC with mural granulosa and theca cells attached to the plate. All samples expressed Lhgr.

In the acute exposure experiment, there was no change in Lhgr expression within the first 6 h of exposure to the different gonadotropin conditions (Fig. 1). After 12 h, Lhgr expression remained low for rFSH10, while high FSH25 increased Lhgr expression (rFSH10 vs rFSH25 and rFSH25+rhCG, P<0.05). In the large antral follicles (rFSH10, day 9), Lhgr was significantly increased by threefold compared with day 6 (rFSH10). On day 9, chronic exposure to HP-hMG25 induced significantly more Lhgr mRNA compared with rFSH10 (P<0.05). rFSH25, rFSH25+rhCG, and rFSH25+rhCG showed intermediate Lhgr expression (Fig. 2).

Acute phase: early response in early antral follicles (day 6) to different gonadotropin regimens for 6 and 12 h

After 6 h of exposure, expression of Cxcl12, Cxcr4, and Hif1a were differentially regulated by the different gonadotropin preparations (Fig. 1). Their expression was not influenced by increasing the FSH dose (rFSH10 vs rFSH25). Cxcl12 expression was decreased if high FSH25 was combined with LH bioactivity (rFSH10 vs rFSH25+rhCG and HP-hMG25, P<0.05). Cxcr4 showed an inverse reaction and was upregulated by high FSH25 combined with LH bioactivity (rFSH10 vs rFSH25+lhCG, rhFS25+hcCG, and HP-hMG25, P<0.05). Hif1a expression was similar in all recombiant gonadotropin treatments but reduced in HP-hMG25 (P<0.05).

After 12 h of exposure, Cxcr4, Cyp19a1, and Nr4a1 showed gonadotropin-dependent expression (Fig. 1). Their expression was significantly increased by increasing the FSH dose (rFSH10 vs rFSH25, P<0.05). If high FSH25 was combined with LH bioactivity, expression of Cxcr4 (rFSH25 vs rFSH25+lhCG, P<0.05) and Nr4a1 (rFSH25 vs rFSH25+hcCG and HP-hMG25, P<0.05) was further increased.

Mok, Lss, Timp1, Vegfa, and Sdc4 expression was not influenced by gonadotropin treatment. The baseline of rFSH10 was upregulated from 0 to 6 h and returned back to initial values at 12 h for Mok, Lss, Timp1, Hif1a, Cxcl12, Cxcr4, and Nr4a1 (Table 2, ANOVA + Tukey, P<0.05).

Chronic phase: continuous exposure to different gonadotropin regimens during antral growth from days 6 to 9

During antral follicle growth in rFSH10 from days 6 to 9, Lhgr was threefold upregulated and Egfr was 1.5-fold downregulated (Fig. 2, t-test, P<0.05). After a 3-day exposure of the growing follicles to different gonadotropin treatments, Cxcr4, Cyp19a1, and Cyp11a1 expression was gradually increasing from rFSH10 to rFSH25, to rFSH25+rhCG, and highest in HP-hMG25. Statistical significance was obtained between rFSH10 and HP-hMG25 (P<0.05) for Cxcr4, Cyp19a1, and Cyp11a1 (Fig. 2). A tendency for increased expression in HP-hMG25 was present for Hsd3b1, Cxcl12, Oxtr, Pgr, and Sdc4 (Table 3).

Steroid production

The immediate effects by different gonadotropin treatments on E₂ production on day 6 showed highest E₂ production/ hour in HP-hMG25 both at 6 h (Table 4, P<0.01 vs rFSH10) and 12 h (tendency). By day 9 of culture, the E₂ concentration of rFSH10 was significantly lower compared with the other four conditions (Table 5, P<0.001). The basal progesterone production was elevated in rFSH25+lh bioactivity compared with rFSH10 and rFSH25 (rFSH10 vs rFSH25+lh, P<0.05). Sixteen hours after the ovulatory trigger, no differences in progesterone production were found between the different gonadotropin preparations (P>0.05, data not shown).
Lhcgr expression in the acute and chronic phase

The increase in FSH at day 6 was followed 12 h later by an induction of Lhcgr expression. This was expected as FSH and estrogen (already increased at 6 h in the spent medium) induce Lhcgr during normal antral follicle growth (Richards et al. 1976, Ikeda et al. 2008). Ovulatory doses of LH are known to downregulate the expression of Lhcgr (Hoffman et al. 1991) already after 4 h (Wang & Menon 2005). This is directly caused by Lhcgr mRNA destabilization through MKV, an immediate effect that can be detected by gene expression as soon as 6 h after the ovulatory stimulus (Wang et al. 2007). Lhcgr downregulation was not observed in the current study using high FSH25 supplemented with 5–35 pM of rLH or rhCG during antral growth and no correlation with Mvk expression was found.

The growth of the follicle from the early (day 6) to the late (day 9) antral stage coincided with more Lhcgr expression. The highest level of Lhcgr mRNA was found after exposure to HP-hMG25 for 3 days. Equal levels of E2 were found in all conditions containing high gonadotropin concentrations; hence, E2 cannot be responsible for higher Lhcgr expression in HP-hMG25.

Acute phase (6 and 12 h): Expression levels of Hif1a, Nr4a1, and Cyp19a1 are influenced by gonadotropins

The increase in Hif1a mRNA expression after 6 h seen in early antral follicles in this study was independent of increasing doses of recombinant gonadotropins but was absent in HP-hMG25. In all conditions, Hif1a returned to

**Discussion**

The effects of FSH, LH, and hCG during antral follicle growth were investigated on genes involved in Lhcgr signaling both immediately after exposure (acute phase) and 3 days after exposure (chronic phase).
baseline levels after 12 h. The transient nature of Hif1a as a rapid response mediator of FSH action (Alam et al. 2009) and the equivalent level of induction of its downstream target Vegfa by all gonadotropin treatments after 6 and 12 h could indicate that the total level of induction of Hif1a had been similar in all conditions but differed in kinetics.

An ovulatory dose of LH/hCG causes an immediate response of Nr4a1 expression within 1 h in granulosa cells (Carletti & Christenson 2009) through cAMP-mediated signaling and returns back to basal levels after 9 h (Wu et al. 2005). This stimulates HSD3B2 (Havelock et al. 2005) and represses Cyp19a1 (Wang & Menon 2005), shifting the steroid

Figure 2 Gene expression results during the chronic exposure for 3 days (from days 6 to 9 of culture) to different gonadotropin regimens. Bars represent mean expression ratios of the specific genes over the Rn18s endogenous control with S.E.M. error bars. Statistical analysis was performed by ANOVA + Tukey posttest on day 9 (separated by full lines from the day 6 result) with different small letters indicating statistical differences with at least P < 0.05. In capital letters, statistics (t-test, P < 0.05) are shown for the regulation of the genes over the two time points (days 6–9) in the rFSH10 condition.

Table 3 Gene expression results for the chronic phase: continuous exposure to different gonadotropin regimens during antral growth from days 6 to 9. Values represent relative expression ratios of the specific gene and the endogenous control Rn18s (mean ± S.E.M.)

<table>
<thead>
<tr>
<th></th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 9</th>
<th>Day 9</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FSH10</td>
<td>rFSH10</td>
<td>rFSH25</td>
<td>rFSH25</td>
<td>HP-hMG25</td>
</tr>
<tr>
<td>Hsd3b1</td>
<td>629 ± 112</td>
<td>812 ± 102</td>
<td>650 ± 82</td>
<td>743 ± 139</td>
<td>754 ± 138</td>
</tr>
<tr>
<td>Cxcl12</td>
<td>1·26 ± 0·24</td>
<td>0·84 ± 0·23</td>
<td>0·59 ± 0·16</td>
<td>1·20 ± 0·35</td>
<td>0·98 ± 0·33</td>
</tr>
<tr>
<td>Oxtr</td>
<td>2·31 ± 0·43</td>
<td>2·59 ± 0·48</td>
<td>3·10 ± 0·57</td>
<td>3·10 ± 0·54</td>
<td>2·93 ± 0·46</td>
</tr>
<tr>
<td>Pgr</td>
<td>0·42 ± 0·10</td>
<td>0·36 ± 0·07</td>
<td>0·32 ± 0·07</td>
<td>0·37 ± 0·05</td>
<td>0·41 ± 0·09</td>
</tr>
<tr>
<td>Sdc4</td>
<td>15·6 ± 5·0</td>
<td>25·5 ± 3·9</td>
<td>23·0 ± 3·5</td>
<td>27·4 ± 4·9</td>
<td>26·9 ± 6·1</td>
</tr>
</tbody>
</table>

Between brackets, the amount of samples analyzed for gene expression is given.
secretion profile from estrogenic to progestagenic. In the current experimental setup on day 6, Nr4a1 showed a late induction at 12 h by FSH further elevated by LH activity, which coincided with an induction of Cyp19a1. FSH is believed to induce a slow but sustained cAMP signal during growth while an ovulatory LH surge would elicit a rapid but transient cAMP signal (Conti 2002). Our findings could suggest that during antral growth, Nr4a1 expression is cAMP mediated through Fshr and mild stimulation of Lhgr.

Steroidogenesis: expression of key genes and steroid production in the acute and chronic phase

Cyp19a1 mRNA induction by high FSH after 12 h and the activity of the present CYP19A1 protein (E2 production, Table 4) was kept intact after administration of a small dose of LH or hCG. In rat, cultured granulosa cells exposed to FSH also showed E2 production within a 6-h period (Daniel & Armstrong 1984), suggesting that FSH in our culture model is indeed both activating the existing CYP19A1 protein and inducing its mRNA expression only by 12 h. The E2 production occurred mainly during the first 6 h and was most evident for HP-hMG. Coincidently, an induction of Mok, Lss, and Timp1 expression was seen after 6 h of exposure, independent of gonadotropins. The temporary depletion of steroids after medium refreshment induced Mok and Lss expression (Wang et al. 2007, Ikeda et al. 2008), providing the necessary precursors for E2 synthesis. Timp1 knockout mice showed increased E2 and decreased progesterone (Nothnick 2000) and TIMP1 stimulated progesterone production in tests (Boujrad et al. 1995). This could make Timp1 another target upregulated to bring the follicular steroidogenic environment in balance after medium refreshment.

After 3 days, exposure to different gonadotropin treatments Cyp19a1 tended to be upregulated by low-dose LH activity. The potential to convert pregnenolone into progesterone was comparable as Hsd3b1 expression was not significantly altered. Cyp19a1, converting androgens into estrogens, was again highly expressed in HP-hMG25. Steroid measurements on day 9 corroborated the gene expression patterns, where the E2 and progesterone content for rFSH25+low-dose LH bioactivity was higher compared with rFSH25 alone. This indicates that the presence of a low dose of LH or hCG bioactivity during follicle growth induced a more active steroidogenesis (Wolfenson et al. 2005), which could be a result of a more active transcription of Cyp11a1 and Cyp19a1. Secondly, the additional supply of precursors through LH-stimulated theca cells, which express Cyp11a1 and Hsd3b1 (Nimz et al. 2009), could also provide an elevated steroid level. Basal progesterone levels in low-dose LH-supplemented conditions remained at least 15 times lower than the progesterone levels 16 h after the ovulatory hCG stimulus, on average 104 μg/l.

Cytokines Cxcl12 and Cxcr4 in the acute and chronic phase

The targets from our selection most influenced by a change in gonadotropin regimen were Cxcl12 and its receptor Cxcr4. An alternative (co)-receptor for Cxcl12 and Sdc4 showed no differences. Cytokines and other immune-related processes have been described during ovulation (Richards et al. 2008). Cytokines also show some gene expression patterns specific for immune-like cells, like Cdc34 antigen, myelin basic protein (Mbp), and Cxcr4 (Hernandez-Gonzalez et al. 2006). Here was shown that Cxcr4 and Cxcl12 are present and regulated by gonadotropins in mural/theca cells already during antral follicle growth. Cxcl12 was elevated in rFSH10/25 at 6 h after exposure to different treatments, while reduced in rFSH25+LH bioactivity. As suggested for Hif1a, this could be a matter of kinetics, where additional LH activity enhances the general activity of the cell and hereby more rapidly recovers from medium change.

Cxcr4 was induced by HP-hMG25 but even more by rFSH25+rLH/hCG at 6 and 12 h. This pointed to a different reaction through the Lhgr if ligands are from recombinant or urinary purified origin. High FSH+LH bioactivity induced already at 6 h a massive induction of Cxcr4; however, Cxcr4 levels only increased under the influence of high rFSH25 alone after 12 h. This suggested a synergy in LHCGR and FSHR signaling, both using cAMP as second messenger in inducing Cxcr4 expression. No clear

Table 4  Estradiol-17β production on day 6 at 6 and 12 h after different gonadotropin exposures (mean ± s.e.m.)

<table>
<thead>
<tr>
<th>Gonadotropin</th>
<th>n 6h</th>
<th>12h</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFSH10</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>rFSH25</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>rFSH25+rhCG</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>HP-hMG25</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5  Estradiol-17β and progesterone content on day 9 (mean ± s.e.m.)

<table>
<thead>
<tr>
<th>Gonadotropin</th>
<th>E2</th>
<th>Progesterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFSH10</td>
<td>13.2</td>
<td>10.2</td>
</tr>
<tr>
<td>rFSH25</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>rFSH25+rhCG</td>
<td>6</td>
<td>5.5</td>
</tr>
<tr>
<td>HP-hMG25</td>
<td>8</td>
<td>2.9</td>
</tr>
</tbody>
</table>

A,B Different letters in a column define statistical differences between treatments (P<0.05, ANOVA+Tukey).

interrelation between Cxcl12 and Cxcr4 expression was found. Preliminary laboratory data suggested a cell-type- and cell-stage-specific expression pattern for both Cxcl12 and its receptors; hence, the presented data in mural cells could be too fragmentarious to reveal complex interrelations.

In the current study, exposure to HP-hMG25 for 3 days generated the highest level of Cxcr4 expression, where the immediate response after 6 and 12 h on day 6 was higher if exposed to high FSH and rLH bioactivity. This suggests that slight differences in hormone composition like in the recombinant or urinary purified hormones could affect downstream signaling kinetics.

**Chronic phase (days 6–9): no differences due to gonadotropin treatment for Oxtr, Pgr, and Egfr**

Three receptors implicated in ovulation and luteinization were studied: Egfr, Oxtr, and Pgr. All three genes were not affected by gonadotropins. Absence of significant Oxtr, Pgr, and Egfr expression induction indicated that mural cells did not express early signs of luteinization (Fujinaga et al. 1994, Clemens et al. 1998, Segers et al. 2012), which was reinforced by the endocrine profile on day 9.

**Differences in expression induced by rLH, rhCG, or urinary purified products**

For all genes in this study, equimolar doses of rLH or rhCG did not lead to differences in response in the early or late antral granulosa cells (t-test, \( P > 0.05 \)). Hence, it could be considered that in an in vitro culture system, rLH and rhCG are activating Lhcgr signaling equally.

However, HP-hMG25 did elicit some different responses compared to the recombinant products. Some tendencies were noticed. Six hours after exposure of early antral follicles to rFSH25 with or without LH activity, six of 11 genes (Mok, Lss, Cxcl12, Cxcr4, Hif1a, and Timp1) were upregulated by the medium refreshment. The exposure to HP-hMG25 attenuated the initial response or accelerated the decrease to basal levels as seen for all conditions at 12 h (Mok, Lss, Cxcl12, Hif1a, and Timp1). Secondly, after 3 days of growth in HP-hMG25, nine of ten of the studied genes (all except Cxcl12) showed a tendency for elevated expression in HP-hMG25 compared with the rFSH25/rLH/rhCG treatments. An overall higher activity status of granulosa cells under HP-hMG treatment has also been described for protein levels: higher levels of cytokines were found in mouse follicle culture in HP-hMG if compared with rFSH treatment only (Foster et al. 2010). The differences in gene expression patterns seen in HP-hMG25 are (most likely) an effect of both differences in the FSH and the LH/hCG component. For FSH, it was already shown that recombinant vs urinary purified FSH contains different subsets of isoforms and that acidity of isoforms influenced capacity to induce meiosis in COC, to stimulate proliferation in granulosa cells (GC) (Barrios–De-Tomasi et al. 2002), and to influence embryonic development when applied in follicle culture (Vitt et al. 2001). Like FSH, both LH (Burgon et al. 1996) and hCG (Lopata et al. 1997) consist out of different isoforms in vitro with different pI and hence implications in bioactivity.

**Conclusions**

Lhcgr was present and regulated by gonadotropin type and dose in early and late antral follicles. A low dose of LH or hCG applied during early antral follicle growth did not induce premature luteinization effects in both gene expression patterns and steroid production. Gene expression responses were identical after rLH or rhCG stimulation, suggesting an equal in vitro biopotency. However, the urinary-derived HP-hMG did systematically show the highest gene induction after 3-day exposure. Nr4a1, Cxcl12, and Cxcr4 were most influenced by introducing gonadotropin treatment in early antral follicles. This study emphasizes the complexity of LHCGR and FSHR signaling during folliculogenesis, where in-depth ligand–receptor interaction studies are needed to understand the origin of the differential expression patterns induced by the different ligands.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-12-0150.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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