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Molecular and Cellular Endocrinology 285 (2008) 62–72

MCE
Molecular and Cellular Endocrinology

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# Mutations of the lutropin/choriogonadotropin receptor that do not activate the phosphoinositide cascade allow hCG to induce aromatase expression in immature rat granulosa cells☆

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Received 30 November 2007; received in revised form 16 January 2008; accepted 23 January 2008

**Abstract**

Using primary cultures of immature rat granulosa cells and adenoviral infections we expressed two mutants of the human lutropin receptor (hLHR) that do not activate the phosphoinositide cascade. One mutant (hLFF) has the extracellular domain of the hLHR and the transmembrane and intracellular domains of the hFSHR. The other (hLHR-L457D) has a leucine to aspartate mutation in residue 457 of transmembrane helix 3.

When expressed in immature rat granulosa cells the hLHR stimulates cAMP and inositol phosphate accumulation, transactivates the epidermal growth factor receptor (EGFR), elicits a transient increase in Akt phosphorylation, and a sustained increase in ERK1/2 phosphorylation but aromatase expression is not enhanced. When expressed at comparable densities, hLFF and hLHR-L457D support cAMP accumulation and transient Akt phosphorylation but do not support inositol phosphate accumulation, EGFR transactivation or a sustained phosphorylation of ERK1/2. Cells expressing either of these two mutants respond to hCG with increased aromatase expression.

We also show that addition of hCG to cells expressing the hLHR antagonizes the effects of hFSH on aromatase expression whereas addition of hCG to cells expressing the hLHR-L457D mutant does not.

These results show that activation of the phosphoinositide cascade is upstream of EGFR transactivation and ERK1/2 phosphorylation and that this pathway is a negative regulator of aromatase expression in granulosa cells.


*Keywords:* Granulosa cells; LH receptor; FSH receptor; Aromatase; EGF; Inositol phosphates; ERK1/2

## 1. Introduction

One of the outstanding questions in the development of the ovarian follicle is why two highly homologous hormone receptor pairs (FSH/FSHR and LH/LHR) elicit divergent effects on ovarian gene expression. Recent studies on the functional properties of the recombinant LHR expressed in immature rat granulosa cells show that these divergent effects of the FSH/FSHR and LH/LHR pairs are likely due to the level of receptor expression and signaling cascades that are activated and not due to the expression of the LHR only in differentiated granulosa cells (Andric and Ascoli, 2006; Bebia et al., 2001; Donadeu and Ascoli, 2005; Zeleznik et al., 2003).

By comparing the actions of hFSH and hCG in primary cultures of immature rat granulosa cells expressing different densities of the recombinant hFSHR or hLHR we have shown that both hormones induce aromatase expression when the density of their cognate receptor is low and neither hormone induces aromatase expression when the density of their cognate receptor is high (Donadeu and Ascoli, 2005). We have also shown that when acting on low or high receptor densities, hFSH and hCG activate the cAMP signaling pathway and induce a rapid and transient phosphorylation of ERK1/2 and Akt (Andric and Ascoli, 2006; Donadeu and Ascoli, 2005) whereas at high receptor densities hFSH and hCG also promote the hydrolysis of phosphatidylinositols and provoke a sustained increase in the phosphorylation of ERK1/2 (Andric and Ascoli, 2006; Donadeu and Ascoli, 2005).

Several investigators, including us, have already shown that the ERK1/2 pathway is a negative regulator of aromatase expression (Andric and Ascoli, 2006; McDonald et al., 2006; Su et al., 2006; Zeleznik et al., 2003). We have also shown that pharmacological inhibitors of the EGF network and ERK1/2

☆ Supported by a grant from the National Institute of Child Health and Human Development (HD-28962).

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0303-7207/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2008.01.018

phosphorylation restore the ability of gonadotropins to induce aromatase expression in immature granulosa cells expressing a high density of receptors (Andric and Ascoli, 2006). The lack of useful experimental manipulations that can be used to inhibit the phosphoinositide signaling cascade has made it difficult to test for its involvement as the first step of the proposed pathway leading to the repression of aromatase induction (Andric and Ascoli, 2006). Here we use two mutants of the hLHR that do not activate the phosphoinositide cascade to directly and conclusively document the importance of this signaling cascade on the transactivation of the EGFR, the sustained phosphorylation of ERK1/2, and the repression of aromatase expression.

## 2. Materials and methods

### 2.1. Materials

Purified hCG (CR-127) and purified hFSH (AFP-5720D) were purchased from the National Hormone and Pituitary Agency (Torrance, CA). Purified recombinant hCG and hFSH were kindly provided by Ares Serono (Randolph, MA). Cell culture medium was obtained from Invitrogen Corp. (www.invitrogen.com). Other supplies and reagents used for granulosa cell extraction and culture were obtained from Sigma–Aldrich Corp. (www.sigmaaldrich.com), BD Biosciences (www.bdbiosciences.com) and Fisher (www.fishersci.com). Molecular biology reagents were obtained from Invitrogen Corp. (www.invitrogen.com) and Roche Diagnostics Corp. (www.roche-applied-science.com). All other chemicals were obtained from commonly used suppliers.

### 2.2. Viruses, plasmids and cells

The mutant named hLFF was constructed using standard PCR strategies and it contains the extracellular domain of the hLHR (amino acid residues 1–363) spliced into the transmembrane, and intracellular domains (amino acid residues 373–695) of the hFSHR. The boundaries of the spliced regions of this mutant correspond to the exact boundaries of a chimera of the rat FSHR and LHR (named LFF) previously characterized in this laboratory (Nakamura et al., 1999). An expression vector for hLHR-L457D was kindly provided by D. Segaloff (Shinozaki et al., 2001). These two constructs were subcloned into the RAPAd adenoviral vector and the recombinant adenoviral particles (Ad-hLFF and Ad-hLHR-LD) were prepared by the Gene Transfer Vector Core at The University of Iowa. The preparation of recombinant adenoviral particles coding for the hLHR (Ad-hLHR), and β-galactosidase (Ad-βgal) have been described (Andric and Ascoli, 2006; Donadeu and Ascoli, 2005).

The methods used to isolate, maintain and infect primary cultures of immature rat granulosa cells have also been previously described (Andric and Ascoli, 2006; Donadeu and Ascoli, 2005). These procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Infections with the different receptor-coding viral constructs were done as indicated in the figure legends but the total amount of virus used for each infection was always adjusted with Ad-βgal to give a constant MOI (multiplicity of infection) of 300.

An expression vector for a C-terminally tagged EGFR-GFP was donated to us by Dr. John Koland of this institution. I-10 cells a clonal strain of Leydig tumor cells that lack LHR (Ascoli, 2007; Shin, 1967), were purchased from the American Type Culture Collection (CCL-83), and maintained in DMEM/F12 medium supplemented with 15% horse serum, 20 mM Hepes and 50 μg/ml gentamicin, pH 7.4.

### 2.3. Binding, second messenger and mRNA assays

The binding of $^{125}$I-hCG was measured during a 4 h incubation with 100 ng/ml of $^{125}$I-hCG (Donadeu and Ascoli, 2005). Cyclic AMP accumulation was measured during a 30 min incubation in cells incubated without or with hCG (100 ng/ml) but in the presence of 1 mM isobutylmethylxanthine, a phosphodiesterase inhibitor (Donadeu and Ascoli, 2005; Hipkin et al., 1995a,b; Steiner et al., 1972). Inositol phosphate accumulation was measured during a 1 h incubation in cells incubated without or with hCG (500 ng/ml) but in the presence of 20 mM LiCl, an inhibitor of the phosphatases that dephosporylate inositol phosphates (Ascoli et al., 1989; Donadeu and Ascoli, 2005; Hipkin et al., 1995a,b; Hirakawa et al., 2002). Epiregulin and aromatase mRNA were quantitated using real-time PCR at the end of a 9 or 48 h incubation, respectively, without or with 100 ng/ml hCG as described elsewhere (Andric and Ascoli, 2006; Donadeu and Ascoli, 2005).

The incubation times and hCG concentrations chosen for binding and the second messenger and mRNA assays were previously shown to result in maximal effects (Andric and Ascoli, 2006; Donadeu and Ascoli, 2005; Hirakawa et al., 2002).

### 2.4. ERK and Akt phosphorylation assay

The methods for measuring ERK1/2 and Akt phosphorylation were as previously described (Andric and Ascoli, 2006; Donadeu and Ascoli, 2005) with a few modifications as follows. The membranes were first probed during an overnight incubation with a 1:5000 dilution of dual phospho-specific p44/p42 (Thr202/Tyr204) ERK1/2 antibody (cat # 9101) or a 1:3000 dilution of a phospho(Ser473) AKt antibody (cat # 9271) from Cell Signaling Technology (www.cellsignal.com). This was followed by a second 1-h incubation with a 1:10,000 dilution of a secondary antibody (cat # 170–6515) covalently coupled to horseradish peroxidase from BioRad (www.biorad.com). The immune complexes were visualized and quantified using Super Signal West femto maximum sensitivity detection system (www.piercenet.com) and a digital imaging system (Eastman Kodak Co.). After detection of phospho ERK1/2 or phospho Akt the membranes were stripped with 50 mM Tris–HCl pH 6.2, 100 mM β-mercaptoethanol, 2% SDS for 20–30 min at 70–80 °C and incubated overnight with a 1:5000 dilution of ERK-2 antibody (cat # sc-154) from Santa Cruz Biotechnology (www.sbct.com). This was followed by a 1 h incubation with secondary antibody and detection of the immune complexes as described above. All the ERK1/2 and Akt phosphorylation data presented were corrected for the amount of total ERK-2 detected.

### 2.5. Bioassay for EGF-like growth factors

This assay was carried out by co-culturing primary granulosa cells expressing the hLHR or mutants thereof and I-10 Leydig cells expressing a GFP-tagged form of the human EGFR (henceforth referred to as test cells).[1] When added to these co-cultures hCG acts only on granulosa cells because the test cells do not express the LHR (Ascoli, 2007; Shin, 1967; Shiraishi and Ascoli, 2007). Thus, any hCG-dependent increase in the phosphorylation of the EGFR-GFP has to arise from extracellular, EGF-like factors that are produced by granulosa cells and act on the test cells. In addition, other potential direct effects of the LHR on the phosphorylation of the endogenous EGFR in granulosa cells would not be detected in this assay because the EGFR-GFP has a higher molecular weight than the endogenous EGFR and they can be readily resolved on SDS gels (Shiraishi and Ascoli, 2007).

I-10 cells were transfected with the EGFR-GFP using Lipofectamine® as described elsewhere (Hirakawa et al., 2002; Shiraishi and Ascoli, 2007). The transfected I-10 cells were trypsinized and plated ($4 \times 10^5$ cells) on 6 well plates already containing $2 \times 10^5$ granulosa cells that had been infected one day earlier with Ad-hLHR, Ad-hLFF or Ad-hLHR-LD as described above. After plating the co-cultures were maintained in 2 ml of growth medium (DMEM/F12 medium supplemented with 1 mg/ml bovine serum albumin, 10 mM Hepes, insulin (1 μg/ml), transferrin (1 μg/ml), selenium (1 ng/ml), pH 7.4) for 8 h. The medium was then replaced with assay medium (DMEM/F12 medium sup-

[1] I-10 cells were chosen as test cells because they do not express the LHR, they can be maintained in the same medium as granulosa cells, they are routinely available in our laboratory, and they can be easily cultured and transfected. The fact that they are Leydig cells is irrelevant to this assay. In theory any other cell line that does not express the LHR and can be transfected with the EGFR-GFP could be used as test cells.