The Differential Effects of the Gonadotropin Receptors on Aromatase Expression in Primary Cultures of Immature Rat Granulosa Cells Are Highly Dependent on the Density of Receptors Expressed and the Activation of the Inositol Phosphate Cascade

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Signaling pathways mediating the divergent effects of FSH and LH on aromatase in immature rat granulosa cells were studied by infecting cells with increasing amounts of adenoviral vectors for the human LH receptor (hLHR) or FSH receptor (hFSHR). Increasing amounts of Ad-hLHR, used at a multiplicity of infection (MOI) of 20 or 200 viable viral particles/cell, increased human chorionic gonadotropin (hCG) binding and hCG-induced cAMP and Akt phosphorylation, but inositol phosphates only increased in response to hCG in cells infected with 200 MOI Ad-hLHR. In contrast, hCG increased aromatase expression in cells infected with 20, but not in cells infected with 200, MOI Ad-hLHR. Cells infected with 20 or 200 MOI Ad-hFSHR showed increased hFSH binding and hFSH-induced Akt phosphorylation, but the hFSH-induced cAMP response was unchanged relative to control cells. However, hFSH was able to stimulate the inositol phosphate cascade in the Ad-hFSHR-infected cells, and the hFSH induction of aromatase was abolished. We also found that activation of C kinase or expression of a constitutively active form of Goq inhibited the induction of aromatase by hFSH or 8Br-cAMP. We conclude that the differential effects of FSH and LH on aromatase in immature granulosa cells are highly dependent on gonadotropin receptor density and on the signaling pathways activated. We propose that aromatase is induced by common signals generated by activation of the FSHR and LHR (possibly cAMP and Akt) and that the activation of the inositol phosphate cascade in cells expressing a high density of LHR or FSHR antagonizes this induction. (Endocrinology 146: 3907–3916, 2005)
been shown that two of the hallmark responses of FSH action (i.e. the induction of aromatase and the LHR) are likely due to differences in the signaling properties of the LHR and the FSHR rather than to their expression at different stages of maturation of the granulosa cells (16, 17). It has also been concluded that the ability of FSH to stimulate aromatase and LHR expression involves the activation of the protein kinase B/Akt signaling pathway (17) in addition to the cAMP pathway (1–3, 11, 12, 18–23).

In the experiments presented here, we used adenovirus-directed expression of hLHR and hFSHR in primary cultures of immature rat granulosa cells to determine the receptor-activated pathways that may account for the divergent effects of FSH and LH in granulosa cells and to determine the effects of receptor density on the signaling properties of each receptor. We tested the hypothesis that the differential effects of FSH and LH on aromatase expression are due to the activation of a common stimulatory signaling pathway involving cAMP and an inhibitory signaling pathway that involves inositol phosphates and is uniquely activated by LH. Rationale for this comes from the observations that addition of human chorionic gonadotropin (hCG) to cells expressing a high density of the LHR is able to stimulate not only cAMP but also inositol phosphates (reviewed in Ref. 24) and that pharmacological activation of protein kinase C (PKC) antagonizes the effects of FSH or cAMP on the expression of aromatase and several other ovarian genes (25–28).

In doing these experiments, we also found, unexpectedly, that the functional properties of the FSHR are modified by the coexpression of increasing densities of the LHR.

Materials and Methods

Plasmids, viruses, and cells

The myc-tagged versions of the hLHR (29) and hFSHR (30) were subcloned into the RAPAd adenoviral vector. A constitutively active mutant of Gαi (Q209L) that has reduced capacity to hydrolyze GTP (31–33) was obtained from the Guthrie Research Center (http://www.guthrie.org/AboutGuthrie/Research/CDNA/) and subcloned in the same vector. The recombinant adenoviral particles (Ad-hLHR, Ad-hFSHR, and Ad-GqQ209L) as well as recombinant adenoviral particles coding for β-galactosidase (Ad-β-gal) were prepared by the Gene Transfer Vector Core of the University of Iowa (http://www.uiowa.edu/~gene/vectors.htm).

Granulosa cells from immature (25 d old) female Sprague Dawley rats that had been treated with diethylestilbestrol (2 mg/d, injected sc in 200 μl sesame oil) for 4 d were collected by ovarian puncture as described elsewhere (20, 35). These procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa. Cells were plated in 24-well plates (0.25 million cells/well) or 12-well plates (0.5 million cells/well) that had been precoated with bovine fibronectin. The cells were plated in 24-well plates (0.25 million cells/well) or 12-well plates (0.5 million cells/well) that had been precoated with bovine fibronectin. The cells were incubated at 37°C in culture medium consisting of DMEM/F12 medium (1:1, vol/vol) containing 10 mM HEPES, 50 μg/ml gentamicin, and 0.1% BSA and supplemented with 0.5 mM isobutylmethylxanthine and then incubated at 37°C for 15 min in 0.25 ml of the same medium. Triplicate wells were then incubated with hFSH (100 ng/ml), hCG (100 ng/ml), or buffer at 37°C for an additional 30 min after which total cAMP (i.e. cells + medium) was extracted and measured by RIA as described below (36). Under these conditions cAMP accumulation attains a maximal or near maximal level within 30 min of hormone addition and remains elevated for at least 4 h (30, 37, 38). The concentrations of hormones used were also chosen to elicit maximal cAMP accumulation (30, 37, 38).

To measure production of cAMP, cells in 24-well plates were washed twice with DMEM/F12 medium (1:1, vol/vol) containing 10 mM HEPES, 50 μg/ml gentamicin, and 0.1% BSA and then placed in 0.5 ml of the same medium containing 4 μCi/ml of [2-3H]myo-inositol (NEN Life Science Products, Boston, MA) during the last 18 h of the postinfection incubation. Cells were then washed twice with DMEM/F12 medium (1:1, vol/vol) containing 10 mM HEPES, 50 μg/ml gentamicin, and 0.1% BSA and supplemented with 20 mM LiCl and incubated in 0.5 ml of the same medium at 37°C for 15 min. This was followed by an additional 60-min incubation in the presence of hFSH (500 ng/ml), hCG (500 ng/ml), or buffer. Inositol phosphates were then extracted and quantitated as described (39). These conditions have been shown to result in maximal or near maximal inositol phosphate accumulation in cells expressing the constitutively activated gonadotropin receptors (30, 37, 38). The concentrations of hormones used were also chosen to elicit maximal inositol phosphate accumulation (30, 37, 38, 40).

Akt phosphorylation assays

Cells in 12-well plates were plated as described above with DMEM/F12 medium (1:1, vol/vol) containing 10 mM HEPES, 50 μg/ml gentamicin, and 0.1% BSA and placed in 1 ml of the same medium during the last 18 h of the postinfection incubation. The medium was then replaced with 0.5 ml of the same medium and the cells were incubated for 30 min in the presence of hFSH (100 ng/ml), hCG (100 ng/ml), or buffer only. At the end of the incubation, the cells were placed on ice, quickly washed twice with 1 ml of cold buffer containing 150 mM NaCl and 20 mM HEPES (pH 7.4), and lysed with 35 μl of lysis buffer [150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycytlate, 0.1% sodium dodecyl sulfate, 1 mM Na3VO4, and 1 mM NaF (pH 7.4)] by gentle rocking at 4°C for 30 min. The resulting lysates were clarified by centrifugation and assayed for protein content using the BCA protein assay kit from Bio-Rad Laboratories Inc. (Hercules, CA). Thirty micrograms of protein from each lysate were then resolved on 12% polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes (40).

This was followed by an overnight incubation of the membranes in a 1:3000 dilution of a phospho-AKT (Ser473) antibody (Cell Signaling Technology Inc., Beverly, MA) followed by a second 1-h incubation with...
a 1:3000 dilution of a secondary antibody covalently coupled to horse-radish peroxidase (Bio-Rad Laboratories). Finally, immune complexes were visualized and quantified using the Super Signal West fermo maximum sensitivity detection system (Pierce Chemical Inc., Rockford, IL) and a digital imaging system (Eastman Kodak Co., Rochester, NY). The effects of hCG and hFSH on the phosphorylation of Akt are transient (41, 42), and our time course experiments (not shown) revealed that a 30-min incubation with hCG or hFSH was optimal for the detection of this response. The concentrations of hormones used for the Akt phosphorylation assays were also chosen to elicit a maximal response (data not shown).

Quantitation of mRNA by real-time PCR

Two days after infection, medium was aspirated and cells were incubated at 37 °C for an additional 48 h with fresh culture medium supplemented with 50 ng/ml testosterone and containing hCG (100 ng/ml), hCG (100 ng/ml), 8-bromoadenosine-cAMP (8Br-cAMP; 1 mM), and/or phorbol 12-myristate-13-acetate (PMA, 100 nm). Total RNA was then extracted using Trizol reagent and chloroform followed by isopropanol precipitation. One microgram of collected total RNA was renatured and transcribed using Superscript III and random primers (p(dN)6), and Aromatase cDNA in the reverse transcription (RT) product was quantitated by real-time PCR (43) using Sybr Green (Sybr Green PCR master mix; Applied Biosystems, Foster City, CA) for amplicon detection and ROX as an internal reference dye. Standard PCR settings (50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min) were used in an ABI Prism 7000 sequence detection system (Applied Biosystems). Specific primers (forward, TCCTCTGTAGGCAGTAATG; reverse, GGCCCGATTCCCAGACAGA) were used for the reaction. The amount of aromatase mRNA was calculated using ABI Prism 7000 system; Applied Biosystems, Foster City, CA) for amplicon detection and ROX as an internal reference dye. Standard PCR settings (50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min) were used in an ABI Prism 7000 sequence detection system (Applied Biosystems). Briefly, a cycle threshold (the first cycle at which a detectable increase in the target amplicon is observed) was obtained for each sample and used to calculate the amount of aromatase mRNA relative to a standard curve (43). Glyceraldehyde-3-phosphate dehydrogenase mRNA was also quantitated in the same samples using specific primers (forward, TGCCAAAGTATGATGCATCAAGAAG; reverse, AGGCCCGATGGCGCTT-TAGT) and used to correct for variations in RNA content among samples. The 48-h incubation chosen for the aromatase expression experiments was chosen based on our data (not shown) and those of others (16, 17, 23) who have previously studied this phenomenon.

Statistical methods

To account for experimental variability within each experiment and end point (except for binding data), all values were calculated relative to the value obtained in cells infected with 200 MOI Ad-β-gal and treated with hFSH (which was taken as 1.0) and are thus shown in the corresponding graphs. Data were then analyzed using the SAS Mixed Procedure (44) and Tukey’s tests were used for comparisons among means. In all cases statistical significance was considered at P < 0.05.

Materials

Purified hCG (CR-127, $>$13,000 IU/mg) and purified hFSH (AFP-5720D) were purchased from Dr. A. Parlow (National Hormone and Pituitary Program, Torrance, CA). Purified recombinant hCG and hFSH were kindly provided by Ares SeroNo (Randolph, MA). 125I-hCG and 125I-hFSH were prepared as described elsewhere (45). Partially purified hCG (3000 IU/mg), purchased from Sigma (St. Louis, MO), and partially purified equine FSH (kindly donated by Dr. George Bousfield, Wichita State University, Wichita, KS) were used only for the determination of nonspecific binding (see above). The cAMP antibody and 125I-cAMP used for the cAMP assays were as described before (46). Cell culture medium was obtained from Invitrogen Corp. (Carlsbad, CA). Other supplies and reagents used for granulosa cell extraction and culture were obtained from Sigma-Aldrich Corp. (St Louis, MO), BD Biosciences (San Jose, CA), or Corning (Corning, NY). 8Br-cAMP, PMA, and testosterone were obtained from Sigma-Aldrich. Molecular biology reagents were obtained from Invitrogen or Roche Diagnostics Corp. (Indianapolis, IN). All other chemicals were obtained from commonly used suppliers.

Results

We chose to use adenoviral vectors to induce the expression of recombinant gonadotropin receptors in immature rat granulosa cells because they have shown to mediate high levels of recombinant protein expression in these cells (90% of infected cells) without adversely compromising cellular function (16, 47). Infection with the Ad-hLHR or Ad-hFSHR constructs resulted in the expression of the recombinant receptor as indicated by increased binding of 125I-hCG or 125I-hFSH, respectively (see Figs. 1 and 5). In addition, the recombinant receptors were functional, as shown by the cAMP, inositol, phosh-Act (Fig. 2, A–C, right panels), and aromatase (see Figs. 3 and 7) responses induced on receptor activation with hCG or hFSH. All of these responses were measured using a saturating hormone concentration (see Materials and Methods) and a single time point. The time points and other experimental conditions selected to measure the different responses are different, and they were chosen to provide optimal conditions for measuring each of these individual responses (see Materials and Methods and individual figure legends).

As expected, primary cultures of immature rat granulosa cells infected with a control adenovirus coding for β-gal (Ad-β-gal) bound 125I-hFSH but did not bind 125I-hCG (Fig. 1, white bars in left and right panels). The binding of 125I-hCG dramatically increased in cells infected with 200 MOI Ad-hLHR (Fig. 1, right panel), and this increase was associated with increases in the hCG-induced cAMP, inositol phosphate, and phosh-Akt responses (Fig. 2, A–C, right panels). 125I-hCG binding (Fig. 1, right panel) in the 20 MOI Ad-hLHR-infected cells was measurable (~0.5 ng/106 cells) but was low, compared with binding in 200 MOI Ad-hLHR-infected cells (~3 ng/106 cells), and it was not statistically different from that in the control cells (~0.05 ng/106 cells). However, the clear hCG-induced cAMP and Akt phosphorylation re-

![Fig. 1. 125I-hFSH and 125I-hCG binding to immature granulosa cells infected with different amounts of Ad-hLHR. Primary cultures of immature granulosa cells were infected with adenoviral constructs coding for Ad-β-gal (β-gal) at 200 MOI, a mixture of hLHR at 20 MOI plus Ad-β-gal (hLHR-MOI 20) at 180 MOI, or Ad-hLHR at 200 MOI (hLHR-MOI 200). The binding of 125I-hFSH (left panel) or 125I-hCG (right panel) was measured 2 d after infection as described in Materials and Methods. Each bar is the mean ± SEM of five to 10 independent experiments. Means within a panel with different letters (a, b) are significantly different from each other (P < 0.05).](https://academic.oup.com/endo/article-abstract/146/9/3907/2500388)
responses observed in the cells infected with 20 MOI Ad-hLHR relative to control cells (Figs. 2, A and C, right panels) reassured us that the 20 MOI viral load was indeed inducing the expression of functional hLHR. We attribute the different results obtained with these three methods to the higher sensitivity of the cAMP and Akt phosphorylation assays. In interpreting these and all subsequent data, it is important to note that the levels of expression of the recombinant LHR in cells infected with the Ad-hLHR are comparable with those attained when the expression of the endogenous LHR is induced by stimulation of these cells with FSH or 8Br-cAMP (35). Immature rat granulosa cells maintained for 3 d under the same culture conditions used here but in the presence of maximally effective concentrations of FSH or 8Br-cAMP bind 1–4 ng of $^{125}$I-hCG/10⁶ cells when binding is assayed under the same conditions used here (35).

As mentioned above, hCG-induced cAMP and phospho-Akt responses were readily measurable in cells infected with 20 or 200 MOI Ad-hLHR (Fig. 2, A and C, right panels). The hCG-induced cAMP response increased as receptor expression increased (Fig. 2A, right panel), but the Akt response was already maximal in cells infected with 20 MOI Ad-hLHR (Fig. 2C, right panel). In contrast, only cells expressing the highest density of LHR (i.e. those infected with 200 MOI Ad-hLHR) responded to hCG with a significant increase in inositol phosphates (Fig. 2B, right panel). This is in agreement with results obtained in a number of LHR-transfected cell lines in which it has been shown that an inositol phosphate response to hCG stimulation is detectable only at high levels of LHR expression (24). A clear effect of hCG on the induction of aromatase expression was detected only after hCG stimulation of 20 MOI hLHR-infected cells (Fig. 3, right panel). The hCG-induced aromatase expression in the cells infected with 200 MOI hLHR was about 85% lower than that detected in the 20 MOI hLHR-infected cells, and it was not statistically different from the aromatase response detected in hCG-stimulated control cells (Fig. 3, right panel).

To gain insight into the signaling mechanisms involved in the effects of the activated recombinant hLHR on aromatase, we compared the signaling and gene expression responses elicited by hCG in Ad-hLHR-infected cells with those elicited by hFSH in Ad-βgal-infected cells, which express the endogenous FSHR (Fig. 1, left panel, white bar). As expected, activation of the endogenous FSHR with hFSH induced increases in cAMP levels and Akt phosphorylation (Fig. 2, A and C, middle panels, white bars). Activation of the endogenous FSHR did not result in any detectable accumulation of inositol phosphates, however (Fig. 2B, middle panel, white bar). The increase in cAMP accumulation was of similar magnitude to that induced by hCG in 200 MOI hLHR-infected cells (Fig. 2A; compare the white bar in the middle panel with the black bar in the right panel), and the increase in phospho Akt was less than that induced by hCG in 20 or 200 MOI hLHR-infected cells (Fig. 2C; compare the white bar in the middle panel with the gray and black bars in the right panel). In addition, the activation of the endogenous FSHR resulted, as expected, in an increase in aromatase mRNA, and this increase was of similar magnitude to the increase in aromatase induced by hCG in the 20 MOI hLHR-infected cells (Fig. 3; compare white bar in the middle panel with the gray bar in the
Fig. 3. hFSH- and hCG-induced aromatase mRNA in immature granulosa cells infected with different amounts of Ad-hLHR. Primary cultures of immature granulosa cells were infected with adenoviral constructs as described in the legend to Fig. 1. Two days after infection cells were incubated with buffer only (left panel), hFSH (middle panel), or hCG (right panel). Total RNA was then collected and used to quantify aromatase mRNA using RT followed by real-time PCR, as described in Materials and Methods. For ease of comparison with other figures and to avoid the inherent variability in absolute values associated with the use of primary cultures, all data are expressed relative to the hFSH-induced aromatase response of cells infected with βgal (see white bar in the middle panel). The levels of aromatase mRNA in cells incubated with buffer only were often undetectable (see left panel). Each bar is the mean ± SEM of four to 11 independent experiments. Means within a panel with different letters (a, b, c) are significantly different from each other (P < 0.05).

(right panel). These data clearly support the hypothesis that aromatase can be induced by a common signal or signals (either cAMP alone or a combination of cAMP and phospho-Akt) generated by activation of the FSHR and LHR and that a second signal selectively generated by activation of high densities of the LHR antagonizes this induction. Our results (Fig. 2B) are also consistent with the hypothesis that the inositol phosphate cascade is this inhibitory signal generated by activation of the LHR when expressed at a high density.

To more directly test the involvement of the activation of the inositol phosphate cascade in the LHR-induced inhibition of aromatase expression, we used Ad-βgal-infected immature granulosa cells to examine the effects of mutational activation of Gq or pharmacological activation of PKC on aromatase induction by hFSH or 8Br-cAMP (Fig. 4). A construct coding for a constitutively active Gq mutant (GqQ209L) that strongly activates the inositol phosphate cascade (48) was used at a viral load that induced levels of inositol phosphates similar to those induced by activation of the hLHR in 200 MOI hLHR-infected cells (Table 1 and Fig. 2B, right panel) and did not affect 125I-hFSH binding or the hFSH-induced cAMP response (Table 1). As shown in Figs. 4 and 8, 8Br-cAMP induced an increase in aromatase mRNA of similar magnitude to that induced by hFSH, and both of these increases were reduced in cells infected with Ad-GqQ209L (Fig. 4). In agreement with previous results (26), we also found that pharmacological activation of PKC with PMA diminished the stimulatory effects of hFSH or 8Br-cAMP on aromatase expression (Fig. 4). Thus, activation of the inositol phosphate cascade by expression of the constitutively active

Gq or activation of PKC (one of the two branches of this cascade) is sufficient to inhibit the induction of aromatase by hFSH or 8Br-cAMP. These results further indicate that the differential effects of FSH and hCG on the induction of aromatase shown in Fig. 3 are mediated by the preferential hLHR-induced activation of the inositol phosphate cascade in cells expressing high densities of hLHR. Furthermore, we

TABLE 1. Effect of infection with Ad-GqQ209L on inositol phosphate, 125I-hFSH binding, and cAMP responses in immature granulosa cells

<table>
<thead>
<tr>
<th>Response measured</th>
<th>Adenoviral construct used for infection</th>
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<tbody>
<tr>
<td>Inositol phosphates accumulation (relative to βgal-infected cells)</td>
<td>βgal 9.8 ± 1.5b</td>
</tr>
<tr>
<td>125I-hFSH binding (ng/10^6 cells)</td>
<td>GqQ209L 1.7 ± 0.1</td>
</tr>
<tr>
<td>FSH-induced cAMP accumulation (relative to βgal-infected cells)</td>
<td>βgal 2.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>GqQ209L 0.9 ± 0.1</td>
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</tbody>
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Immature granulosa cells were infected with 200 MOI of Ad-βgal or a mixture of 0.5 MOI of Ad-GqQ209L and 199.5 MOI of Ad-βgal. The assays shown were performed 2 d after infection as described in Materials and Methods.

a For the inositol phosphate assays, the Ad-βgal-infected cells were stimulated with hFSH but the Ad-GqQ209L-infected cells were incubated with buffer only.

b A statistically significant difference (P < 0.05) between the Ad-βgal- and the Ad-GqQ209L-infected cells.

c 125I-hFSH binding and hFSH-induced cAMP accumulation were measured as described in Materials and Methods. The results are not statistically different (P > 0.1) between Ad-βgal- and Ad-GqQ209L-infected cells.
conclude that this effect is mediated (at least in part) by the activation of PKC because it can be reproduced by pharmacological activation of this kinase.

Because the expression of increasing densities of the hLHR in immature granulosa cells could potentially affect the expression and signaling properties of the endogenous FSHR, it is also important to investigate this possibility. This is an important issue because endogenous LHR expression increases as granulosa cells differentiate in vivo (49), and the effects of this increase in LHR density on signaling by the coexpressed endogenous FSHR have not been studied. Interestingly, our data show that expression of increasing densities of the recombinant hLHR affects some aspects of hFSH-induced signaling in these cells. Increased expression of the hLHR had no effect on the binding of 125I-hFSH to the endogenous FSHR (Fig. 1, left panel) or the hFSH-induced phospho-Akt response (Fig. 2C, middle panel). There was, however, an approximately 25% reduction in the hFSH-induced cAMP response in 200 MOI hLHR-infected cells relative to Ad-βgal-infected cells (Fig. 2A, middle panel) as well as a reduction in the hFSH-induced aromatase mRNA in cells infected with 20 MOI hLHR or 200 MOI hLHR (41 and 87%, respectively; Fig. 3, middle panel). Thus, the expression of the recombinant, nonactivated hLHR directly or indirectly impaired the ability of hFSH to induce aromatase mRNA, and this seemed to involve, at least partially, a decrease in FSHR-stimulated cAMP production.

Because the density of the recombinant hLHR expressed in 200 MOI Ad-hLHR infected cells is 3- to 4-fold higher than the density of the endogenous FSHR (compare the white bar in the left panel and the black bar in the right panel of Fig. 1), it was also important to determine whether the functional properties of the FSHR change as the density of the FSHR increases. This issue was addressed by analyzing the actions of hFSH and hCG in immature granulosa cells infected with increasing amounts of an adenovirus coding for the hFSHR (Figs. 5–7). Infection with 20 or 200 MOI Ad-hFSHR resulted in a 1.5- and 16-fold increase in 125I-hFSH binding, respectively, to Ad-βgal-infected cells, which express only the endogenous FSHR (Fig. 5, left panel). These increased levels of hFSH had no effect on the hFSH-induced cAMP response (Fig. 6A, middle panel), but they elicited a detectable increase in the hFSH-induced inositol phosphate (Fig. 6B, middle panel), and they enhanced the hFSH-induced phospho-Akt response (Fig. 6C, middle panel). Although the magnitude of the phospho-Akt response observed in cells expressing the recombinant hFSHR is similar to that mediated by the recombinant hLHR (compare the middle panel of Fig. 6C with the right panel of Fig. 2C), the magnitude of the inositol phosphate response is lower than that mediated by the recombinant hLHR, especially when receptor density is taken into account. Thus, whereas granulosa cells infected with 200 MOI of Ad-hLHR bound approximately 3 ng 125I-hCG/10⁶ cells (Fig. 1, right panel) and responded to hCG with an approximately 4-fold increase in inositol phosphates (Fig. 2B, right panel), cells infected with 20 or 200 MOI of Ad-hFSHR bound approximately 4 or approximately 16 ng 125I-hFSH/10⁶ cells, respectively (Fig. 5, left panel) and responded to hFSH with only an approximately 2-fold increase in inositol phosphate accumulation (Fig. 6B, middle panel). Interestingly, at these high levels of hFSHR expression, the ability of hFSH to induce aromatase expression was drastically reduced when compared with the cells infected with Ad-βgal (Fig. 7, middle panel) despite the unchanged hFSH-induced cAMP response (Fig. 6A, middle panel) and the enhanced phospho-Akt response (Fig. 6C, middle panel). Thus, these results are also consistent with the hypothesis that activation of the inositol phosphate cascade (which in this case is mediated by engaging the increased levels of the recombinant hFSHR with hFSH) is responsible for a decrease in the ability of hFSH to induce aromatase expression.

Finally, the effects of Ad-hFSHR infection on endogenous LHR expression and signaling were evaluated in immature rat granulosa cells. As shown in the right panels of Figs. 5–7, the increased expression of hFSH did not affect 125I-hCG binding or hCG-induced cAMP, inositol phosphate, phospho-AKT, or aromatase mRNA.

Discussion

The identification of the signaling mechanisms underlying the differential effects of FSH and LH on granulosa cells has been considerably limited by the expression of the endoge-
nous LHR and FSHR at different stages of granulosa cell differentiation. Thus, when studying the signaling pathways activated by the endogenous gonadotropin receptors present in granulosa cells, a distinction cannot be made between differences in signaling due to receptor type or changes in the intracellular signaling milieu, i.e. steroidogenic enzymes and signaling proteins, that may occur as a consequence of the differentiation of the granulosa cells. The observation that high levels of recombinant protein expression can be induced in rat immature granulosa cells by using adenoviral vectors (47) obviated this problem because it allowed for the expression of the recombinant LHR in these cells (16, 17). Here we used adenoviral vectors to express the recombinant hLHR and hFSHR in immature rat granulosa cells and study the effects of both receptor type and receptor density on signaling pathways and aromatase expression. In agreement with the results of others (16), we conclude that the differential actions of the LH/CG and FSH on granulosa cells are not due to the stage of cell differentiation. Importantly, our results show that the effects of LHR and FSHR activation on aromatase expression in immature granulosa cells are highly dependent on receptor density and signaling pathways activated. We show that, when expressed at low densities, the activated hLHR can induce aromatase expression to a similar extent as that observed when the endogenous FSHR is activated. When expressed at high densities, however, the activated hLHR is unable to induce aromatase expression. This finding, that the effects of hCG on aromatase expression are dependent on the density of the hLHR expressed, is in agreement with previous data.
showing that a biphasic aromatase response occurs with increasing doses of hCG in cultured differentiated granulosa cells expressing the endogenous LHR (21, 23, 51). In this system low concentrations of hCG stimulate aromatase expression to a similar extent as a maximally effective concentration of FSH, whereas higher concentrations of hCG do not stimulate aromatase expression. Our findings underscore the physiological importance of the progressive increase in the density of LHR that occurs during granulosa cell differentiation (49).

The data presented here also show for the first time that with increasing densities of hFSHR, the induction of aromatase by the activated FSHR declines substantially (Fig. 7). Seven studies (15, 52, 53) reported that ovulatory surge doses of FSH have LH surge-like effects in estradiol-primed, hypophysectomized rats or mice, i.e. they induce follicular luteinization and ovulation. Thus, as for the LHR, opposing effects in granulosa cells result from low FSHR densities/low FSH concentrations and high FSHR densities/high FSH concentrations. Because the type of signaling pathways activated by GPCRs may vary as the density of receptors or the amount of agonist used increase (54), we hypothesize that at low signal strengths (i.e. low receptor density and/or agonist concentration), the LHR and FSHR activate a common signaling pathway (or pathways) that is/are stimulatory to the expression of aromatase and that at high signal strengths (i.e. high receptor density and/or agonist concentration), they also activate (similar or distinct) signaling pathways that are inhibitory to the expression of aromatase.

The adenylyl cyclase/cAMP pathway is likely to represent a common pathway activated by the LHR and FSHR that is involved in the induction of aromatase. All investigators agree that the LHR and FSHR activate this signaling system (6–10) and that addition of cAMP analogs, or the receptor-independent activation of the cAMP signaling system, can induce the expression of aromatase in granulosa cells (1–3, 11, 12, 18, 19, 21–23). The data presented here are consistent with this hypothesis. When exposed to a maximally effective concentration of hFSH, the density of endogenous FSHR present in immature granulosa cells is already high enough to mediate maximal cAMP accumulation and is optimal for hFSH-induced aromatase expression. An increase in the density of FSHR elicited by infection with Ad-hFSHR does not increase the magnitude of the hFSH-induced cAMP response, but it substantially reduces the induction of aromatase by hFSH (Figs. 5–7), presumably because of the simultaneous activation of an opposing pathway. When immature granulosa cells are infected with Ad-hLHR, the optimal induction of aromatase by hCG occurs at a low density of recombinant hLHR when the magnitude of the hCG-induced cAMP response is lower than that induced by hFSH acting through the endogenous FSHR (Figs. 1–3). As the density of the recombinant hLHR increases, the magnitude of the hCG-induced cAMP response also increases, but the hCG-induced aromatase expression decreases substantially (Figs. 1–3), again, because of the presumed, simultaneous activation of an opposing pathway.

In view of our results and those of others (17, 41, 42), one must also consider the possibility that the phosphorylation of Akt represents an additional, common pathway activated by the LHR and FSHR that, together with cAMP, is involved in the induction of aromatase. Others have previously shown that injection of hCG results in Akt phosphorylation in the ovaries of rats in diestrus (42), and we have shown here that hCG increases Akt phosphorylation in immature granulosa cells expressing increasing densities of the recombinant hLHR (Fig. 2C). FSH, acting through the endogenous FSHR, has also been reported to increase Akt phosphorylation (41, 55). We have confirmed those results here and have also shown that increased expression of the recombinant hFSHR enhances this response (Fig. 6C). The involvement of the Akt pathway on the FSH-induced expression of aromatase is supported by the ability of a dominant-negative form of Akt to diminish this effect of FSH (17). In addition, Akt overexpression was shown to potentiate the induction of aromatase by FSH, cAMP, and a constitutively active form of the LHR (17).

Because there is general agreement that the inositol phosphate cascade is activated when the LHR is expressed at high densities and exposed to high concentrations of hCG (see Figs. 1 and 2 and Ref. 24) and because the activation of this pathway is inhibitory to the cAMP- and FSH-induced aromatase expression (Fig. 4 and Ref. 26), we propose that the LH/CG-induced activation of the inositol phosphate cascade is responsible for the decrease in aromatase induction observed at high densities of the LHR (Fig. 3). This proposal is consistent with the proposed role of PKC activation (a downstream effect of the activation of the inositol phosphate cascade) as a mediator of the changes in aromatase and other ovarian genes that occur at high densities of the LHR (i.e. during luteinization; see Refs. 26, 28, 56, and 57). It should also be noted that although a relationship between PKC activation and inhibition of aromatase expression has been shown in granulosa cells (26), our results show for the first time a direct association between the activation of the inositol phosphate cascade (induced here by expression of a constitutively active Gαq; see Fig. 4) and the suppression of aromatase. This finding is important because although there is agreement about the ability of LH/CG to activate the inositol phosphate cascade, there are conflicting reports on the ability of LH/CG to activate PKC (37, 58). More studies are therefore needed to determine which of the two second messengers are responsible for the inhibition of the cAMP- and FSH-induced aromatase expression. Lastly, as noted here, high densities of the FSHR also result in a substantial suppression of the hFSH-induced aromatase expression without a decrease in hFSH-induced cAMP accumulation (Figs. 5–7). Interestingly, this suppression of aromatase expression is also associated with a modest increase in the ability of hFSH to stimulate the inositol phosphate cascade. Therefore, it is possible that this hFSH-induced activation of the inositol phosphate cascade is also responsible for the suppression of aromatase expression that occurs at high levels of FSHR expression.

More definitive proof for the proposed role of the inositol phosphate cascade as an inhibitor of aromatase expression at high densities of the LHR or FSHR can be obtained only by showing that inhibition of the inositol phosphate cascade
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FSHR and LHR (either cAMP accumulation alone or a combined response to a common signals generated by activation of the signaling pathways involved in the induction of aromatase). We propose that aromatase expression is increased by the recombinant hLHR because it is observed in cells that have not been exposed to hCG. Moreover, because the increase in the hFSH-induced cAMP response in cells expressing high densities of hLHR is small (Fig. 2A), it appears unlikely that this decrease would be solely responsible for the large decrease in aromatase induction (Fig. 3). This finding needs to be further investigated, but several explanations may be considered. First, because we know that GPCRs in general (59, 60) and the LHR in particular (40) have some intrinsic level of basal activity, it is easy to envision how an increase in the density of the recombinant hLHR may increase the basal activation of signaling pathways that impair the ability of the FSHR to induce aromatase expression. This is especially true if the signaling pathways involved in the induction of aromatase (such as cAMP and/or phospho-Akt) are already maximally or near maximally activated by engaging the endogenous FSHR. The reduced signaling ability of the endogenous FSHR in cells expressing high LHR densities may also result from increased dimerization between the endogenous FSHR and the recombinant hLHR. Precedence of this exists with other GPCRs such as the different subtypes of opioid receptors (61, 62). Finally, we (63) and others (64, 65) have reported that the LHR and FSHR can each associate with at least one distinct protein that affects signal transduction. Thus, the increased expression of either receptor could bring increasing amounts of these associating proteins to the plasma membrane in which they could affect homologous or heterologous signaling.

In summary, we show that the differential effects of FSH and LH on aromatase in immature granulosa cells are highly dependent on gonadotropin receptor density and the signaling pathways activated. We propose that aromatase is induced by common signals generated by activation of the FSHR and LHR (either cAMP accumulation alone or a combination of cAMP accumulation and Akt phosphorylation) and that activation of the inositol phosphate cascade in cells expressing a high density of LHR or FSHR antagonizes this induction.

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