Direct evidence for de-novo synthesis of LH receptors in cultured pig granulosa cells in response to FSH

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Summary. FSH plus insulin, cortisol, and thyroxine (IFT) stimulated incorporation of dense isotope-containing (^2H , ^{13}C , ^{15}N) amino acids into soluble ^{125}I -labelled hCG binding sites. Evidence of new synthesis of binding sites appeared as early as 3 h after the beginning of the pulse-labelling period. By 48 h the majority of detectable soluble ^{125}I -labelled hCG binding sites appeared to be newly synthesized. Studies with FSH + IFT and puromycin indicated that FSH + IFT stimulated synthesis of new LH/hCG binding sites, and that internalization or degradation of LH/hCG binding sites may also require protein synthesis.

Introduction

The dramatic morphological and biochemical changes which occur in granulosa cells in response to FSH provide a suitable system to study the mechanism of protein hormone induction of cellular differentiation and maturation. One of these changes is the increase in numbers of LH/hCG binding sites (Channing & Kammerman, 1973; Kammerman & Ross, 1975) in response to FSH (Zelevnik, Midgley, & Reichert, 1974; Channing, 1975). A period of 24–48 h is required for FSH induction of functional binding sites in porcine granulosa cells (Loeken & Channing, 1981). Addition of 10^{-6} M-puromycin or cycloheximide or 0.5 μg actinomycin D/ml blocks induction of binding sites (Loeken, 1982). These observations suggest that FSH induces expression of genes for proteins involved in luteinization including the LH/hCG binding site. We have examined this possibility in the present study.

Materials and Methods

Pig ovaries were obtained from a local meat packing plant. Granulosa cells were aspirated from small (1–2 mm) follicles and cultured as previously described (Channing & Ledwitz-Rigby, 1975). Cells were cultured in Medium 199 + 10% pig serum (both from Grand Island Biological Co., Grand Island, New York) ± 0.1 μg NIH-FSH-S15/ml (20 U/mg, from the National Pituitary Agency, containing <0.005 U LH activity/mg) + insulin (1 mU/ml), cortisol (0.01 $\mu\text{g}/\text{ml}$) and thyroxine (10^{-7} M). Insulin, cortisol and thyroxine (IFT) were always added with the FSH because they potentiate the FSH effects on LH/hCG binding site induction and progesterone secretion. The doses of IFT used have no stimulatory effects upon LH/hCG binding sites in the absence of FSH (Channing *et al.*, 1982). At the times indicated, media were aspirated and replaced with fresh medium containing the same hormones as at time zero, or medium consisting of amino acid-deficient Medium 199 + 10% serum with or without FSH + IFT and 1.5 mg heavy isotope-containing (^2H , ^{13}C , ^{15}N) amino acids/ml. Any deficient amino acids in the heavy isotope amino acid mixture were adjusted to within 10% of the concentration contained in Medium 199 with light

isotope-containing amino acids. Media enriched in heavy isotope-containing amino acids will be referred to as 'dense media', and light isotope-containing amino acid media (normal Medium 199) will be referred to as 'light media'. Because both types of medium contained 10% serum composed of light isotope-containing amino acids and proteins, an accurate determination of the actual proportion of heavy or light isotope-containing amino acids in each experiment was not performed. Cells were cultured in light media for at least 24 h because culture for more than 24 h is required before the effects of FSH on induction of LH/hCG binding sites are observed (Loeken & Channing, 1981) but culture over extended times in dense media decreased LH/hCG binding site levels. Unless stated otherwise, dense amino acids produced from an algal hydrolysate were purchased from Merck, Sharp, and Dohme Isotopes, Montreal, Canada. These mixtures were 99% ^2H , 90% ^{13}C , and 99% ^{15}N , or 90% ^{13}C and 99% ^{15}N . In one experiment, a dense amino acid preparation kindly provided by Dr Daniel Lane (The Johns Hopkins School of Medicine, Baltimore, MD) was used. After culture, about 5×10^6 cells were pooled and frozen for later assay of ^{125}I -labelled hCG binding and DNA, and media were saved for radioimmunoassay of progesterone concentration (Channing, Tsai & Sachs, 1976). The cells (1 ml) were suspended in 0.1 M-Tris + 1% BSA, pH 7.4, and incubated with 0.5 ml of 5×10^5 c.p.m. ^{125}I -labelled hCG for 1 h at 37°C with shaking. Unbound radioactivity was removed by centrifugation at 10 000 g and washing with 0.1 M-Tris + 0.1% pig skin gelatin, pH 7.4; this was repeated 3 times. Cells then were solubilized with 1 ml 1% Triton X-100 for 30 min on ice after glass pestle homogenization. The soluble binding sites were subsequently clarified by centrifugation for 30 min at 170 000 g. The extracted radioactivity was receptor-associated for at least 24 h at 4°C as determined by chromatography on Sepharose CL-6B (Pharmacia, Uppsala, Sweden). Solubilization of bound ^{125}I -labelled hCG was routinely 33% for uninduced binding sites (those obtained from granulosa cells from small follicles not cultured with FSH) to about 60% for light or dense amino acid-containing, FSH + IFT-induced binding sites (those obtained from granulosa cells cultured in the presence of FSH + IFT). The soluble receptor preparation (0.5 ml) was mixed with 1.7 ml of a solution of CsCl (0.568 g/ml; Bethesda Research Laboratories, Bethesda, MD) as described by Reed & Lane (1980), and was centrifuged in a Beckman SW50.1 rotor for 18 h at 300 000 g. During each centrifugation controls of only free ^{125}I -labelled hCG or only light amino acid-containing receptors bound to ^{125}I -labelled hCG were run to determine their sedimentation positions in CsCl gradients. Fractions were subsequently collected (50–75 μl) from the bottom of each tube and counted for 3–5 min in a γ -counter (LKB Rackgamma, LKB Instruments) with 67% counting efficiency for ^{125}I . Gradient profiles shown in Text-figs 1–5 are of specifically bound solubilized ^{125}I -labelled hCG-binding site complexes (total c.p.m. minus non-specific LH-saturated background level c.p.m.). Experimental results displayed in Text-figs 1–5 were verified by repetition 1–3 times. Results were qualitatively reproducible. No statistical analyses were performed on repeated experiments because these experimental procedures provided qualitative rather than quantitative information. DNA was measured spectrofluorometrically (Brunk, Jones, & James, 1979).

Results

Synthesis of LH/hCG binding sites

There were low but detectable levels of soluble LH/hCG binding sites on granulosa cells recovered fresh from small pig follicles at 0 h (Text-fig. 1a). Culture for 96 h with FSH induced higher levels of binding sites in both the detergent-insoluble (not shown) and detergent-soluble fractions (Text-fig. 1a–d). When cells were grown in dense amino acid-enriched media + FSH + IFT, there was a gradual shift in the peak of ^{125}I -labelled hCG binding sites toward the dense region of the CsCl gradient (Text-fig. 1b–d). Binding sites from cells cultured for the entire 108-h period in only light amino acid-containing media eluted in the same position of the gradient as did light binding sites recovered at 0 h (Text-fig. 1a). Binding sites from cells which were grown for 48 h

Text-fig. 1. Gradient profiles of specific ^{125}I -labelled hCG binding sites solubilized from pig granulosa cells grown in medium with light ($\times$, open circles) or dense (^2H , ^{13}C , ^{15}N , closed circles) isotope-containing amino acids. Elution positions of the peak of free ^{125}I -labelled hCG (fraction 8) and light receptor-bound ^{125}I -labelled hCG (fraction 14) peaks are indicated by arrows and lines, respectively. Binding sites were solubilized using fresh granulosa cells recovered from follicles (a) or cells cultured for 96 h in light amino acid-containing media plus FSH + IFT, with 1 medium change occurring at 48 h, plus 3 (b), 6 (c), or 12 (d) additional hours in dense media or 12 h in light media (a) plus hormones. Fractions (75 μl) were collected from the bottom of each gradient and counted for 3–5 min in a γ counter.

in dense media exhibited the maximum density shift, distinct from those grown in light media by 4 fractions, and very few binding sites eluted in the light region of the gradient (Text-fig. 2).

The morphology of the cultured cells, checked by phase-contrast microscopy, and double stranded DNA content did not appear to differ whether the cells were grown in dense or light media, and yet cells cultured in dense media exhibited less LH/hCG binding with time than did cells cultured in light media. There was also a 45% decrease in [^3H]leucine incorporation into protein during culture in dense amino acids. Nevertheless, these lower numbers of binding sites on cells cultured in dense media may still be adequate for functional luteinization, as indicated by the ability of LH/hCG binding sites to secrete progesterone in response to LH. When cells on which LH/hCG binding sites had been induced in dense amino acid-containing media + FSH + IFT were cultured for 2 days in light amino acid-containing media $\pm$ 1 mU NIH-S23-LH/ml, LH-treated cells secreted 275.4 ± 21.9 ng progesterone/ 5×10^5 cells per 48 h while non-LH-treated cells secreted 116.5 ± 5.4 ng/ 5×10^5 cells. This was a 2.4-fold stimulation of progesterone secretion by LH. Similarly, cells cultured the entire time in light media secreted 181.1 ± 11.2 ng progesterone/ 5×10^5 cells per 48 h without LH and 292.25 ± 20.9 ng/ 5×10^5 cells per 48 h with LH stimulation. Thus, LH/hCG binding sites induced by FSH + IFT treatment in dense media appear to be as responsive to LH with regard to progesterone secretion as those induced in light media ($P < 0.05$) as shown by t test analysis.

Text-fig. 2. Gradient profiles of soluble LH/hCG binding sites from pig granulosa cells grown in light (open circles) or dense (^2H , ^{13}C , ^{15}N , closed circles) amino acid-containing media. Culture conditions were similar to those used in Text-fig. 1 except that cells were grown in light medium for 48 h followed by culture in light or dense medium for an additional 48 h. Positions of the peaks of dense and light receptor-bound hCG (fractions 9 and 13, respectively) are shown, and a free ^{125}I -labelled hCG was at fraction 7. Fractions (75 μl) were collected.

Decay of LH/hCG binding sites

Only cells cultured in the presence, but not in the absence of FSH + IFT, had detectable LH/hCG binding sites (Text-fig. 3a). These experiments were performed within 48 h of harvesting granulosa cells from small follicles which is early in the induction process; hence the number of LH/hCG binding sites is low. The duration of these experiments was necessarily short because we have found that exposure of cells to 10^{-5} M-puromycin for more than 48 h inhibits cell viability (Loeken, 1982). The buoyant density of the binding sites increased when cells were grown in 0 or 10^{-7} M-puromycin, a dose which inhibits protein synthesis by about 5% (Loeken, 1982). Very little ^{125}I -labelled hCG binding activity in light or dense regions of the gradient was observed in extracts of cells cultured with 10^{-6} M-puromycin (Text-fig. 3c) which inhibits protein synthesis by about 50%. When cells were cultured with 10^{-5} M-puromycin, which inhibits about 95% of protein synthesis, both control and FSH + IFT-treated cells exhibited higher LH/hCG binding site levels than did cells cultured with 10^{-6} M-puromycin, but virtually all of these binding sites eluted in the light region of the gradient.

Dependence of LH/hCG binding site synthesis on FSH

To obtain further evidence that FSH + IFT stimulates new LH/hCG binding site synthesis rather than inhibiting degradation of binding sites which may be synthesized at a constant rate in the presence or absence of FSH + IFT, the effect of removal of FSH + IFT from culture on binding site synthesis was examined. Cells were cultured for 2 days in light amino acid-containing

Text-fig. 3. Gradient profiles of LH/hCG binding sites solubilized from cells grown in dense (^2H , ^{13}C , ^{15}N) amino acids in the presence (closed circles) or absence (open circles) of FSH + IFT $\pm 10^{-7}$ – 10^{-5} M-puromycin. Positions of free ^{125}I -labelled hCG (fraction 8) and ^{125}I -labelled hCG bound to light binding sites (fraction 15) are indicated by arrows and lines, respectively. Cells were grown for 24 h in light amino acid-containing media with or without FSH + IFT. Media were then replaced with dense amino acid-containing media including the same hormones as the first 24 h $\pm$ puromycin. Fractions (75 μl) were collected and counted.

media with FSH + IFT, then media were replaced for a further 24 h with light or dense media. Both types of media contained additional FSH + IFT or no hormone. As shown in Text-fig. 4, when FSH + IFT was removed the cells exhibited less soluble LH/hCG binding than did cells exposed the whole time to FSH + IFT in light or dense amino acid-containing media. Furthermore, cells cultured in dense media without FSH + IFT did not exhibit the shift in density of LH/hCG binding sites which occurred on cells cultured with FSH + IFT.

Aggregation of soluble LH/hCG binding sites

During the time course of pulse-labelling in dense amino acid-containing media, there was a gradual density shift in the peak of hCG binding activity, rather than a clearly distinguishable dense peak which appeared and a light peak which disappeared as have been observed with insulin

Text-fig. 4. Gradient profiles of LH/hCG binding sites from cells cultured for 2 days in light amino acid-containing media with FSH + IFT followed by culture for 2 days in (a) dense or (b) light media with (O) or without (x) FSH + IFT. Elution positions of the peak of dense binding sites (fraction 25) and light binding sites (fraction 28) are indicated by lines. Fractions (60 μl) were collected and counted.

and epidermal growth factor receptors (Reed & Lane, 1980; Krupp, Connolly, & Lane, 1982). With a resolution of dense and light peaks of at least 4 fractions, this should theoretically have been possible. When LH/hCG binding sites solubilized from cells grown for 48 h in dense amino acids were bound to ^{125}I -labelled hCG and binding sites isolated from cells grown in light amino acids were bound to ^{131}I -labelled hCG, centrifugation of each preparation separately resulted in appearance of two distinct peaks (Text-fig. 5). However, when light and dense binding sites were mixed and centrifuged in the same tube, the peaks became broader and less distinct, perhaps due to formation of aggregates. The possibility that these binding sites form multimeric aggregates during solubilization is supported by our observation that solubilized LH/hCG binding sites elute from CL-6B chromatography as a complex with a molecular weight greater than 370 000, the upper limit of the estimated molecular weight of LH/hCG binding sites (Ji, Yoo, Kaltenbach & Ji, 1981).

Discussion

The results displayed in Text-figs 1 and 2 demonstrate that culture of pig granulosa cells with FSH + IFT induced newly synthesized LH/hCG binding sites. Replacement of most of the previously synthesized binding sites with a newly synthesized pool appeared to take place within 48 h. Two additional sets of experiments demonstrated that FSH + IFT stimulated new LH/hCG binding site protein synthesis and did not simply inhibit degradation of binding sites synthesized at a constant rate. First of all, only cells cultured in the presence, but not the absence, of FSH + IFT exhibited increases in newly synthesized LH/hCG binding sites (Text-fig. 3a,b). This response was

Text-fig. 5. Gradient profiles of ^{125}I -labelled hCG bound to binding sites solubilized from cells grown in dense (^2H , ^{13}C) amino acids (●) and ^{131}I -labelled hCG bound to binding sites from cells grown in light media (○). Cells were grown as described in Text-fig. 2. The mixed gradient contained $450\ \mu\text{l}$ ^{125}I -labelled hCG-bound binding sites + $50\ \mu\text{l}$ ^{131}I -labelled hCG-bound binding sites. Separate gradients contained $500\ \mu\text{l}$ ^{125}I -labelled hCG-bound receptors (peak density = 1.3695) or ^{131}I -labelled hCG-bound receptors (peak density = 1.3660). Fractions ($60\ \mu\text{l}$) were collected. Data are expressed as % peak radioactivity after subtracting 10% spillover of ^{131}I activity into the ^{125}I channel. A value of 100% ^{125}I binding represented 309 c.p.m. in the mixed gradients and 305 c.p.m. in the separate gradient; for ^{131}I binding it was 1163 c.p.m. and 3765 c.p.m. for the mixed and separate gradients, respectively. The efficiency of ^{131}I counting was 49.2% and of ^{125}I was 36.8% (Packard Autogamma Scintillation Spectrometer).

inhibited by increasing concentrations of puromycin, a protein synthesis inhibitor (Text-fig. 3c,d). Secondly, the appearance of newly synthesized LH/hCG binding sites required continuous exposure to FSH + IFT (Text-fig. 4). That the level of LH/hCG binding decreased after removal of FSH + IFT from the culture suggests that FSH + IFT may be necessary to prevent the degradation of or perhaps to stimulate recycling of previously formed LH/hCG binding sites. These may be additional effects of FSH + IFT which contribute to the induction process, although stimulation of new binding site synthesis is also apparent. If the mechanism of the induction process were only inhibition of degradation of binding sites synthesized at a constant rate, then binding sites from cells cultured in dense amino acid-containing media after removal of FSH + IFT should have exhibited a shift in density similar to that exhibited by binding sites from cells cultured in dense amino acid-containing media with FSH + IFT. We therefore conclude that FSH + IFT stimulated new LH/hCG binding site synthesis. Whether FSH + IFT does, in addition, prolong the half-life of these binding sites could not be ascertained.

That FSH induces new LH/hCG binding site synthesis has been suggested by us and others (Loeken, 1982; Segaloff & Limbird, 1983). However, the previous experiments involved cycloheximide or puromycin inhibition of FSH induction of LH/hCG binding sites and lacked direct evidence of new binding site synthesis. Moreover, Segaloff & Limbird (1983) concluded that because they could not detect an increase in ^{125}I -labelled hCG binding to the Triton X-100-soluble portion of cells after solubilization, there cannot be a preformed intracellular pool of LH/hCG binding sites and increased LH/hCG binding after FSH treatment must therefore require new binding site synthesis. However, other explanations of their data are that there is an intracellular pool of preformed LH/hCG binding sites which is not extractable with Triton X-100, or presynthesized binding sites may require some post-translational modification to allow binding to ^{125}I -labelled hCG. Also, because there was no demonstration of ^{125}I -labelled hCG binding to

granulosa cells before culture, it is possible that FSH acts by decreasing degradation of LH/hCG binding sites synthesized at a constant rate.

Incorporation of dense amino acids into newly synthesized receptors for acetylcholine, insulin, thyroid hormone, and glucocorticoids has been reported (Devreotes, Gardner & Fambrough, 1977; Krupp & Lane, 1981; Raaka & Samuels, 1981, 1983). Our interest in utilizing this approach to study LH/hCG binding sites originated from a desire to understand the cellular mechanisms of protein hormone-stimulated cellular maturation. If we could obtain evidence that FSH + IFT stimulated new synthesis of a protein which is known to appear at higher levels during granulosa cell maturation, namely the binding site for LH/hCG, then effects of hormonal stimulation on transcription or translation could be examined.

There was a decrease in LH/hCG binding sites after culture in dense amino acids. Others have also observed a decrease in receptors for thyroid and glucocorticoid hormones and for insulin after culture in dense amino acid-containing medium (Reed & Lane, 1980; Raaka & Samuels, 1981, 1983). It appears that pig granulosa cells may be particularly sensitive to inhibitory effects of dense amino acids. Since less LH/hCG binding is observed after culture in dense than in light amino acid-containing media plus FSH + IFT, we cannot say that there is no other process in addition to new binding site synthesis involved in the induction process which might be inhibited by exposure to dense amino acids.

We attempted to determine the source of an inhibitory substance in the dense amino acid mixture. Inhibition could not be attributed to ^2H itself, as suggested by Pollack, Tate & Cook (1981), or to any contaminating peptides from the algal hydrolysate. There is probably some unidentifiable contaminant which is not detected by amino acid analysis which inhibits general cell metabolism to which pig granulosa cells are particularly sensitive. However, the inhibition of porcine granulosa cell metabolism by dense amino acids might not have significantly affected their ability to undergo functional luteinization, since cells on which LH/hCG binding had been induced with FSH + IFT during culture in light or dense media were equally responsive to LH with regard to stimulation of progesterone secretion.

The lack of resolution of LH/hCG binding sites into two clearly distinguishable peaks was unexpected but not unprecedented. It is possible that our LH/hCG binding sites consist of monomers which sediment as aggregates containing a mixture of light and dense binding sites as in the case of the 10S tetrameric glucocorticoid receptor (Raaka & Samuels, 1983). Aggregates of LH/hCG binding sites may form after solubilization. As shown in Text-fig. 5, dense binding sites were more clearly resolved from light binding sites when they were centrifuged separately; when centrifuged together, the ^{131}I -labelled light binding sites appeared to sediment as a broader peak with increased radioactivity sedimenting toward the dense region of the gradient. Therefore, this apparent aggregation made precise calculations of the rates of binding site synthesis and degradation impossible.

That protein synthesis may be required for internalization or degradation of LH/hCG binding sites was suggested by our observation that cells cultured with or without FSH + IFT plus 10^{-5} M-puromycin exhibited significantly greater binding of ^{125}I -labelled hCG than if grown in the presence of 10^{-6} M-puromycin (Loeken, 1982). This is supported by the results in Text-fig. 3. Cells cultured with 10^{-5} M-puromycin with or without FSH exhibited more LH/hCG binding than did cells cultured with 10^{-6} M-puromycin, but these binding sites eluted predominantly in the light region of the gradient. This suggests that new synthesis of LH/hCG binding sites can be inhibited by concentrations of puromycin of 10^{-6} M or greater and that synthesis of another protein(s) responsible for binding site internalization or degradation can be inhibited by 10^{-5} M-puromycin. Protein synthesis has also been implicated in the turnover of insulin receptors and sheep luteal LH receptors (Kosmakos & Roth, 1980; Suter & Niswender, 1983).

In conclusion, we believe that this is the first demonstration that FSH + IFT stimulates de-novo LH/hCG binding site synthesis. Disappearance of previously synthesized LH/hCG binding sites during culture without FSH + IFT suggests that FSH + IFT is required to maintain as well as

to increase the number of LH/hCG binding sites. Whether other processes are also operative to increase further the number of binding sites has not been determined. Also a protein(s) with a short half life which is inhibited by 10^{-5} M-puromycin may regulate receptor turnover. This suggests that, after FSH + IFT stimulation, synthesis and turnover of LH/hCG binding sites are controlled by complex molecular control mechanisms involving new protein synthesis and perhaps also RNA synthesis.

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